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Mort Cellulaire et Maladie de Parkinson : Rôle de la Synphiline-1, de la Parkine et de DJ-1

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Avant Propos

La première description de la maladie de Parkinson remonte à 1817, date à laquelle un médecin britannique du nom de James Parkinson rédigea un livre « An essay on the shaking palsy ». Il y décrivit, le cas de six patients présentant des tremblements involontaires des membres variant en intensité, et le cas de cinq patients ayant une propension à courber le dos, ainsi qu'à passer de la marche à la course. Il associa ces symptômes à une seule et même maladie qu'il nomma « Shaking Palsy ». Plus tard à la fin du XIXème siècle, le Dr Jean Martin Charcot, désigna cette maladie comme un syndrome et décida de lui donner le nom de Parkinson.

La maladie de Parkinson est un syndrome moteur très invalidant pour les patients, qui reste rare avant 50 ans. Son incidence dans les pays occidentaux en fait le second syndrome neurodégénératif après la maladie d'Alzheimer. Il se déclare généralement de façon sporadique autour de 60 ans avec une prévalence de 1 à 2 cas pour 1000. Cette prévalence augmente avec l'âge, en effet, on passe de 6 à 8 cas pour 1000 entre 65 et 69 ans, à 26 à 35 cas pour 1000 entre 85 et 89 ans. Il existe également des formes dites familiales ou génétiques, elles représentent environ 5% des cas, parmi elles les formes dites juvéniles sont plus précoces (entre 30 et 45 ans) et plus sévères.

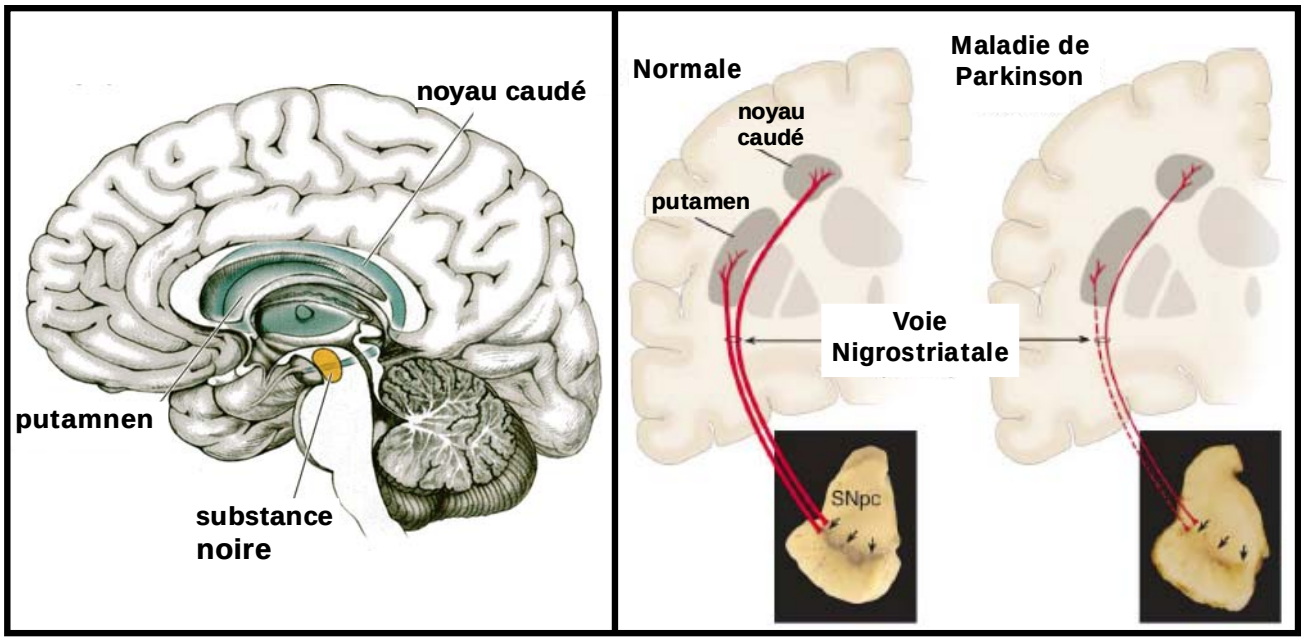
La maladie de Parkinson est caractérisée par la dégénérescence spécifique d'une partie du système nerveux central : les neurones dopaminergiques de la substance noire pars compacta (locus niger) par apoptose. La disparition des neurones produisant la dopamine, neurotransmetteur essentiel dans le contrôle des mouvements, entraîne une akinésie, une perte progressive des capacités motrices, et l'apparition de mouvements incontrôlés chez les patients. Cette dégénérescence neuronale est associée à la présence d'inclusions cytoplasmiques, les corps de Lewy, que l'on retrouve typiquement dans la substance noire ainsi que dans le locus

coeruleus. De part son évolution lente, la maladie de Parkinson reste asymptomatique durant plusieurs années.

Lorsque les premiers symptômes moteurs apparaissent, le traitement majoritairement utilisé, la L-Dopa, permet aux malades de vivre quasiment normalement. Ce traitement devient inefficace lors de la phase tardive. Il existe actuellement d'autres thérapies, cependant, tous les traitements utilisés ne sont pour l'instant que des traitements palliatifs.

En l'absence de traitement curatif, il est essentiel de chercher à comprendre les mécanismes cellulaires et moléculaires qui conduisent à la dégénérescence des neurones dopaminergiques. L'étude des formes familiales est une approche intéressante, elle a conduit à l'identification de protéines mutées, impliquées dans le développement de la maladie. Notamment, elle a permis de mettre en évidence des modifications et des dysfonctionnements moléculaires conduisant à l'exacerbation des processus cellulaires impliqués dans l'apoptose. Dans ce contexte, je me suis consacrée à l'étude fonctionnelle de la synphiline-1, DJ-1 et de la parkine, des protéines directement ou indirectement impliquées dans les formes génétiques de la maladie, afin de mieux connaître leurs fonctions physiologiques et l'influence des mutations pathogènes, dans le déroulement des processus apoptotiques. Parallèlement, je me suis également attachée à l'étude de différentes voies de régulation contrôlant ces protéines, ainsi qu'à l'étude des interactions physiques et fonctionnelles pouvant exister entre elles.

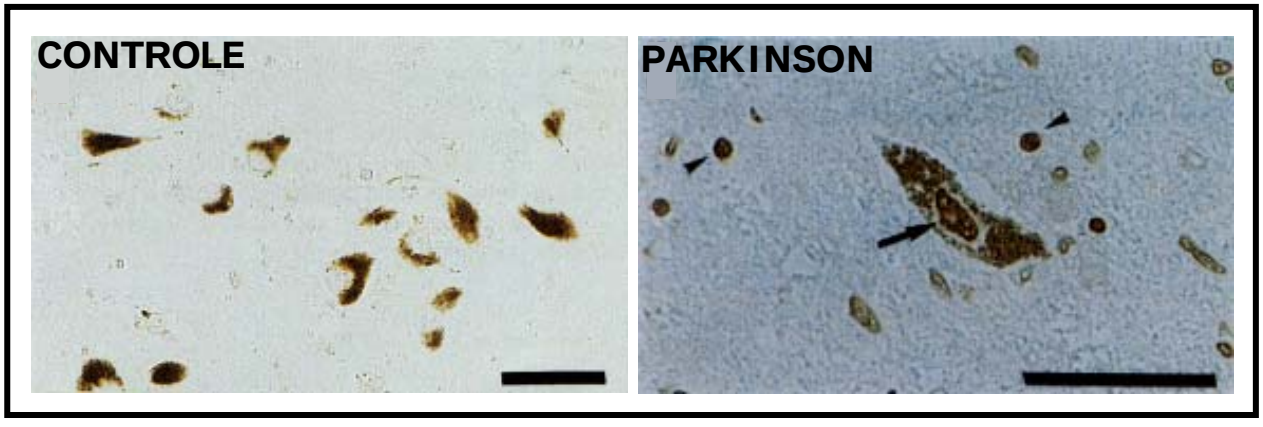
A) Introduction



D'après Dauer W., et al, Neuron (2003) Vol. 39

Figure 1: La voie nigro-striatale en condition normale et en condition pathologique

A gauche les différents noyaux composants la voie nigro-striatale.
A droite le circuit reliant la substance noire au putamen et au noyau caudé



D'après Mochizuki H., et al, J Neurol Sci. (1996) Vol. 137

Figure 2: Section de cerveau de patient parkinsonien marqué par TUNEL.

Marquage *in situ* par la méthode TUNEL de l'ADN fragmenté des noyaux des neurones de la substance noire en apoptose.

A.I) Histopathologie

Aujourd'hui, on considère que la maladie de Parkinson est due à la perte du système de transmission dopaminergique, accompagnée, dans les stades plus tardifs de celle des systèmes noradrénergique, sérotoninergique et cholinergique. Cette dégénérescence est accompagnée de l'apparition d'inclusions cytoplasmiques appelées corps de Lewy. Ces différents marqueurs histopathologiques permettent en outre d'assurer la véracité du diagnostic.

A.I.1) Dégénérescence multiple :

Chez les patients parkinsoniens, les premiers symptômes moteurs n'apparaissent que lorsque l'on observe une perte de près de 60 à 80% des neurones dopaminergiques. La dépigmentation de la substance noire permet de rendre compte de la disparition des neurones dopaminergiques contenant des pigments de neuromélanine (Figure 1) (Dauer and Przedborski, 2003). Les fibres nerveuses projetant de la substance noire vers le putamen et le noyau caudé, sont directement affectées par la forte diminution du taux de dopamine produit par les neurones dopaminergiques, ce qui conduit à une perte progressive du contrôle moteur (Figure 1). Les mécanismes contribuant à la perte progressive des neurones dopaminergiques de la SNpc, sont mal connus. Cependant, nous savons que cette dégénérescence est principalement due à une exacerbation des phénomènes d'apoptose (Figure 2) (Mochizuki et al., 1996).

La disparition du système dopaminergique, s'accompagne d'une dégénérescence progressive d'autres voies nerveuses telles que les systèmes noradrénergique (au niveau du locus coeruleus) (Remy et al., 2005), cholinergiques (au niveau noyau basal

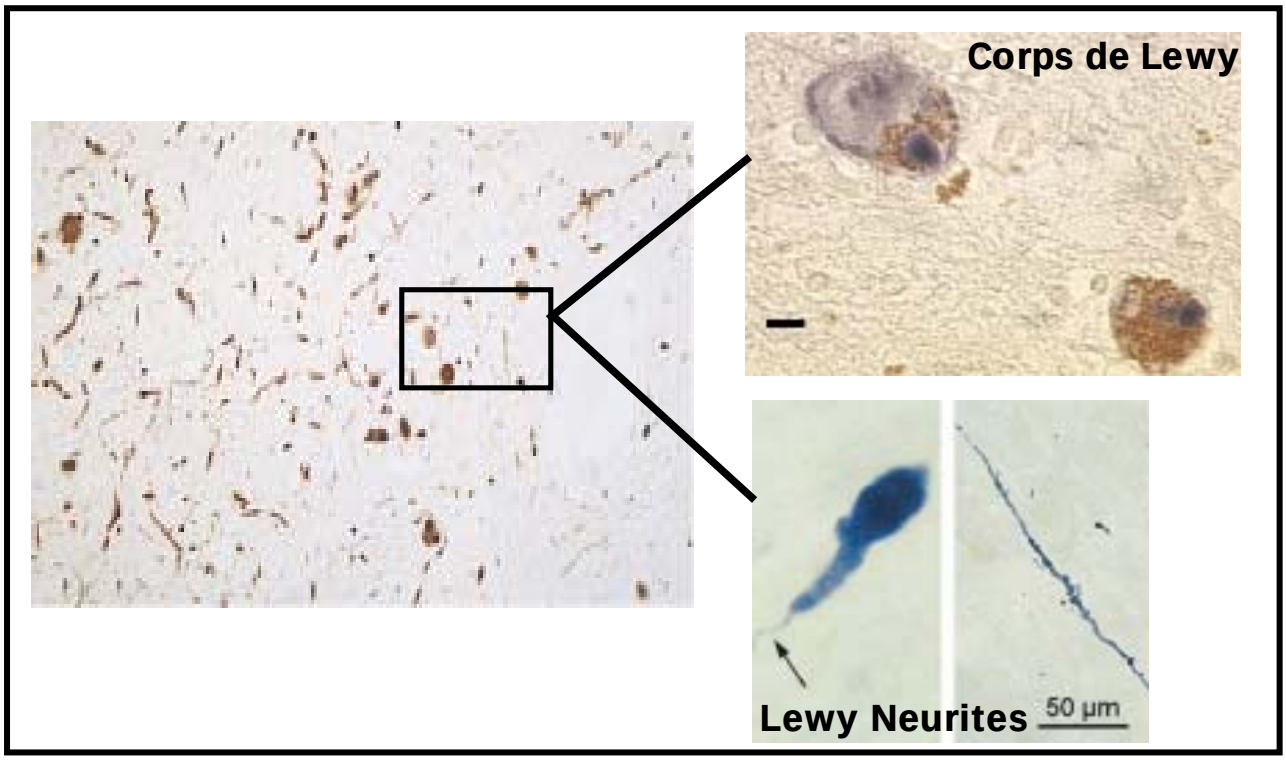


Figure 3 : Les corps et les neurites de Lewy

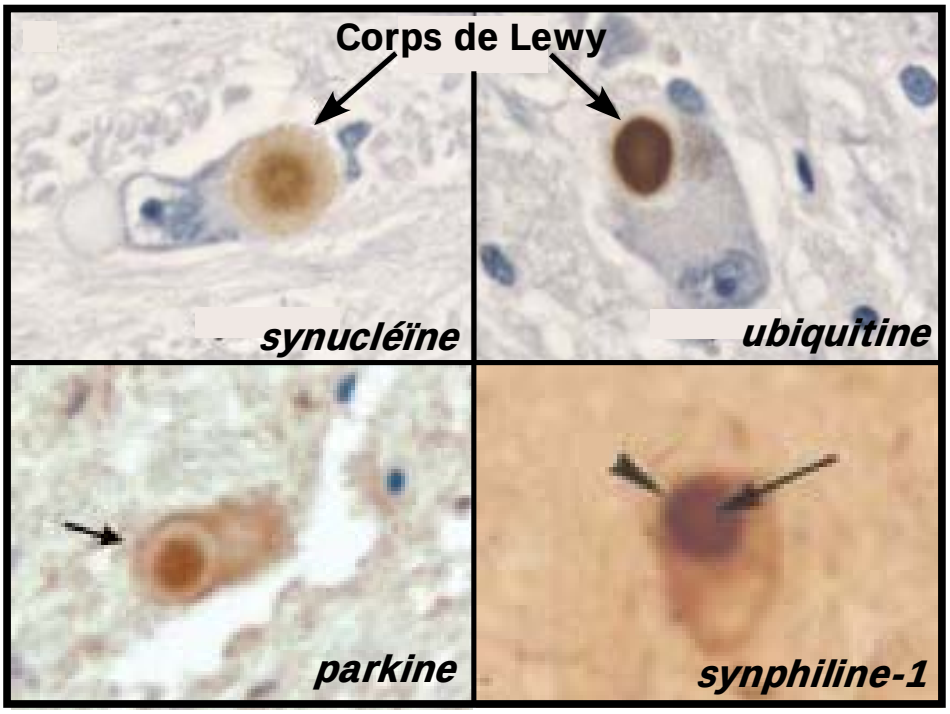


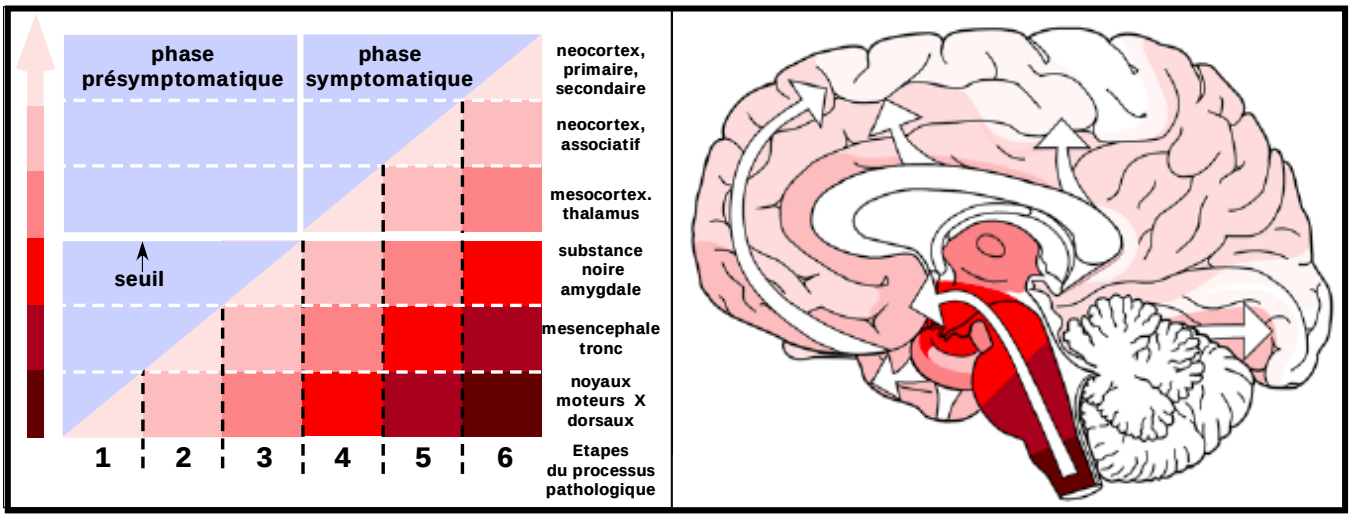
Figure 4 : Immunomarquages de différents composants des corps de Lewy

de Meynert) (Spehlmann and Stahl, 1976), et sérotoninergiques (au niveau du raphé) (Doder et al., 2003; Murai et al., 2001). Le cortex cérébral, le bulbe olfactif et l'hippocampe font aussi partie des structures atteintes dans les phases tardives de la maladie. C'est l'atteinte des nombreuses voies de neurotransmission qui entraîne l'apparition des symptômes dits non moteurs.

A.I.2) Les Corps de Lewy et les neurites de Lewy:

En 1912, F.H. Lewy a décrit pour la première fois des inclusions cytoplasmiques présentes dans les noyaux neuronaux de patients atteints par la maladie de Parkinson (Lewy 1912) (Holdorff, 2002). On les nomme aujourd'hui corps de Lewy lorsque ces inclusions ont une forme globulaire, et neurites de Lewy lorsqu'elles ont une forme de fuseau (Figure 3).

On retrouve ces inclusions dans d'autres pathologies neurodégénératives telles que la Démence à corps de Lewy ou encore dans les AMS « atrophie multi-systématisée ». Les corps de Lewy sont des inclusions intracytoplasmiques principalement constitués de protéines agrégées. Quelle que soit la maladie dans laquelle on les observe, le composant majeur des corps de Lewy est l'α-synucléine (Spillantini et al., 1997). Cette caractéristique en fait des maladies classées sous le nom de synucléinopathies. Actuellement, plus de 70 protéines ont été identifiées dans les corps de Lewy (Wakabayashi et al., 2007). Il est intéressant de noter que parmi ces 70 protéines on retrouve les protéines impliquées dans les formes familiales de la maladie de Parkinson : la parkine (Schlossmacher et al., 2002), PINK1 (Gandhi et al., 2006), DJ-1 (Bandopadhyay et al., 2004), l'α-synucléine (Spillantini et al., 1997), UCHL1 (Lowe et al., 1990), LRRK2 (Miklossy et al., 2006) et la synphiline-1 (Wakabayashi et al., 2000) (Figure 4).



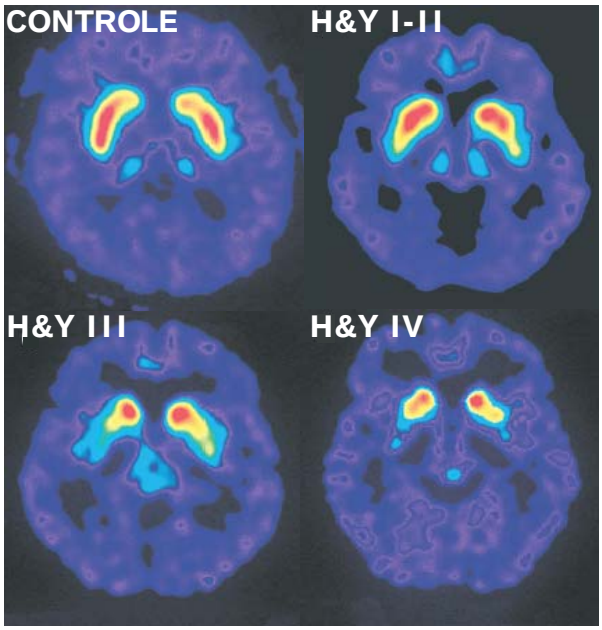
D'après Braak H., et al, Cell Tissue Res (2004) Vol. 318

Figure 5 : Schématisation de la progression des marques histopathologiques

Le schéma de gauche illustre l'évolution des lésions dans les différentes parties du cerveau, au cours des deux phases du développement de la maladie de Parkinson. L'illustration de droite permet de visualiser cette évolution.

L'examen des cerveaux de patients atteints par la maladie de Parkinson, montre la présence de corps de Lewy dans de nombreuses zones également touchées par la dégénérescence, le raphé, le cortex cérébral, le bulbe olfactif ou encore l'hippocampe (Braak et al., 2003; Braak et al., 2006) (Figure 5). Le rôle des corps de Lewy porte encore à controverse. Leur présence dans les zones de dégénérescence (substance noire), leur densité dans le cortex corrélée avec l'évolution des défauts cognitifs, et leur nombre plus élevé chez les patients ayant une perte neuronale moins importante comparée aux patients ayant une mort neuronale sévère, suggèrent que ces inclusions sont neurotoxiques (Takahashi and Wakabayashi, 2001),(Terry, 2000). La diminution, lorsqu'il y a formation d'inclusions cytoplasmiques, de la toxicité induite par certaines protéines agrégées (α-synucléine ou la synphiline-1A), suggèrent que les corps de Lewy sont neuroprotecteurs (Ding et al., 2002; Lashuel et al., 2002; Liani et al., 2004; Tanaka et al., 2004). Ils pourraient permettre le stockage dans un même espace de protéines agrégées afin de diminuer les dommages qu'elles pourraient induire dans la cellule.

Stades	Symptômes
1	- Signes et Symptômes d'un seul coté - Symptômes légers - Symptômes gênant mais pas invalidant - Présente généralement tremblement d'un membre - Changement dans la posture, la démarche et l'expression faciale
2	- Symptômes bilatéraux - Incapacités minimes - Posture et démarche affectées
3	- Ralentissement significatif des mouvementes du corps - Premiers défaut de l'équilibre en marche ou statique - Dysfonctionnement générale qui est modérément sévère
4	- Symptômes sévères - Peut toujours marché mais de façon limitée - Rigidité et Bradykinésie - N'est plus capable de vivre seul - Les tremblements peuvent être moins importants
5	- Etape chaotique - Invalidité complète - Ne peut ni rester debout ni marcher - Requière des soins permanant



D'après http://mediwire.sma.org/main

Figure 6 : Echelle de Hoehn et Yahr en parallèle avec des scanners de patients

Cette figure permet de mettre en parallèle l'échelle de Hoehn et Yahr qui classent les différents symptômes dans 5 stades et la disparition des neurones dopaminergiques visibles sur des scanners de patients.

A.II) Les symptômes cliniques :

En 1967, Hoehne et Yahr ont établi une échelle clinique de progression des symptômes chez les patients, qui découpe en 5 étapes l'évolution de la maladie vers l'invalidité totale (Figure 6). Elle permet d'établir une corrélation entre l'évolution des symptômes et la disparition des neurones dopaminergiques ainsi que la propagation des corps de Lewy (Figures 5). Il existe d'autres échelles mises en place par la suite comme l'« Unified Parkinson's Disease Rating Scale (UPDRS) » qui permettent d'établir un diagnostique plus précis. Les symptômes cliniques de la maladie de Parkinson sont classés en deux catégories distinctes, le parkinsonisme moteur et le parkinsonisme non moteur.

A.II.1) Les symptômes moteurs :

A.II.1.a) Le tremblement au repos :

Le tremblement au repos est le symptôme le plus connu de la maladie de Parkinson. Environ 70% des malades en sont affectés. Comme le décrivent Hoehn et Yarh, le tremblement commence par être unilatéral, le plus souvent au niveau de la main ou du pied. Ces tremblements se produisent uniquement au repos. Ils diminuent lors de mouvements volontaires jusqu'à parfois disparaître, ce qui les différencie des tremblements occasionnés, par exemple, par la prise de médicament, ou apparaissant au cours d'autres maladies neurologiques comme le syndrome du tremblement essentiel. Le tremblement au repos devient bilatéral avec la progression de la maladie.

A.II.2.b) La bradykinésie :

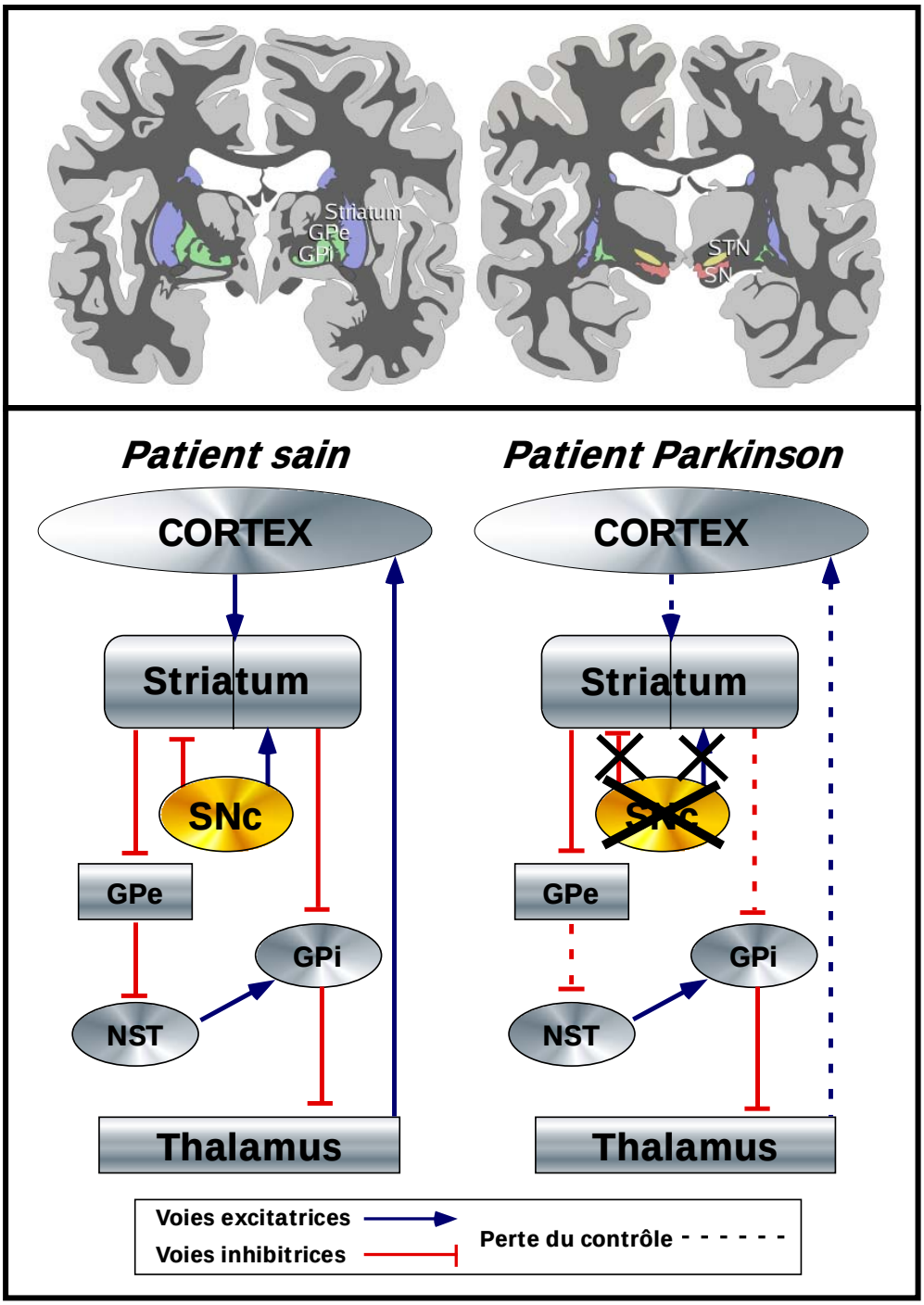
La bradykinésie se caractérise par une lenteur lors de l'exécution des mouvements volontaires. Elle peut toucher tous les membres, mais aussi la face, la voix devient faible et monotone. L'un des signes clinique est une marche lente composée de pas lents et courts. La bradykinésie rend les actes de la vie quotidienne pénibles, par exemple, le ralentissement des mouvements de la main et des doigts entraîne la perte des mouvements fins comme l'écriture. On peut aller de la bradykinésie jusqu'à la disparition totale du mouvement ou akinésie.

A.II.2.c) L'akinésie :

L'akinésie se caractérise par une lenteur dans l'initiation des mouvements, et par une diminution de toute forme d'activité motrice, avec une forte tendance à l'immobilité. La perte d'activité motrice comprend les mouvements volontaires, les mouvements associés, les mouvements d'ajustement postural, les mouvements d'expression gestuelle et émotionnelle mais aussi les mouvements automatiques inconscients (le patient doit commander consciemment la plupart de ses mouvement auparavant inconscients).

A.II.1.d) La rigidité musculaire :

Les patients définissent la rigidité musculaire, comme étant une raideur musculaire - par exemple, au niveau du cou et des épaules - ce qui entraîne une posture courbée. Lors des examens cliniques, on observe des mouvements passifs et saccadés. Lors de l'exercice de la marche, elle se caractérise souvent par la diminution du balancement des bras. Cette rigidité se traduit aussi par une raideur des muscles de la face, et donc une diminution de la capacité des malades à



D'après Alexander G., et al, Trends Neurosci. (1990) Vol. 13

Figure 7 : Schéma du circuit des ganglions de la base chez un patient sain et un parkinsonien

SNc : substance noire pars compacta ; GPe/i : Globus pallidus externe ou interne ; NST : noyaux sous-thalamiques

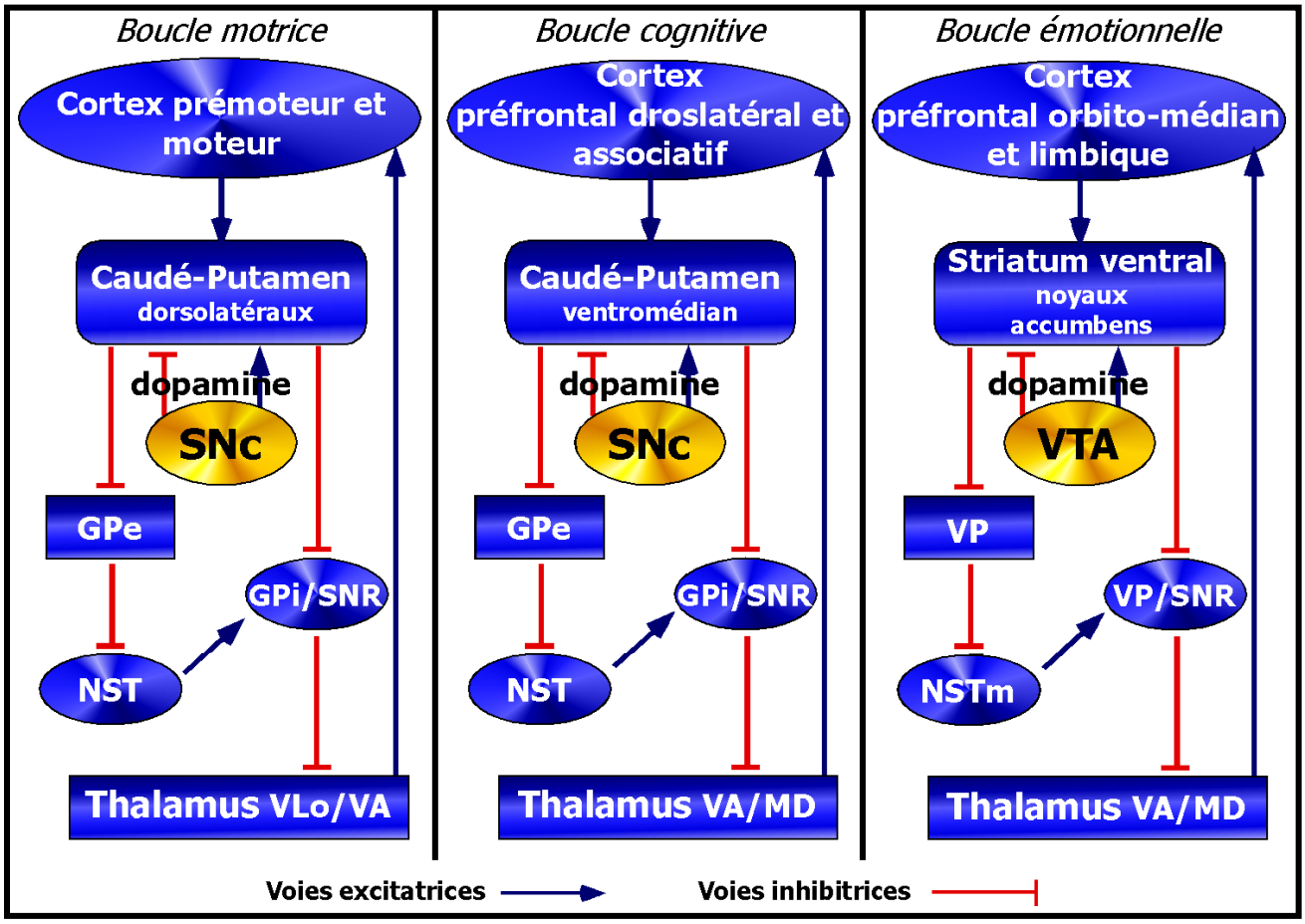
communiquer ; c'est ce que l'on nomme une hypomimie. Cette rigidité contribue aussi bien souvent aux douleurs musculaires.

A.II.1.e) L'instabilité posturale :

L'instabilité posturale survient plus tardivement que les autres symptômes musculaires. Elle se manifeste par des troubles de l'équilibre ; les patients ont des difficultés à démarrer la marche, la démarche devient piétinante, le corps est voûté, les bras ne se balancent plus. Les pas sont incontrôlés et peuvent s'accélérer brusquement, le patient semble courir pour s'empêcher de tomber, ce que l'on nomme festination. Le Dr. Armand Trousseau disait : « Ils semblent courir après leur centre de gravité ». Les patients ont également une forte tendance à tomber vers l'avant (propulsion ou antépulsion) ou vers l'arrière (rétropulsion). Ces troubles de l'équilibre provoquent des chutes de plus en plus fréquentes au fur et à mesure de l'évolution de la maladie.

D'un point de vue physiologique, la chute du niveau de dopamine provoque la perte des tonus inhibiteur et excitateur de la dopamine, provoquant le dérèglement de la boucle des ganglions de la base responsables du contrôle du mouvement (Figure 7).

La boucle motrice n'est cependant pas la seule boucle dans laquelle la dopamine joue un rôle prépondérant. Elle intervient également dans le contrôle des boucles cognitive (circuit associatif et cognitif) et émotionnelle (circuit limbique) (Figure 8), boucles impliquées dans l'apparition de symptômes non-moteurs.



D'après Wolters E., Parkinsonism and related disorders (2007)

Figure 8: Représentations schématiques de différentes boucles impliquant le circuit des ganglions de la base

On peut visualiser ici les trois boucles nerveuses dans lesquelles les ganglions de la base interviennent. L'atteinte des ces voies nerveuses chez un patients parkinosniens induits les symptômes dits non-moteurs.

SNc/SNr : substance noire pars compacta/pars reticula ; GPe/i : Globus pallidus externe/ interne ; NST : noyaux sous-thamiques (m: médian) ; VTA : aire tegmentale ventrale ; VP : pallidium ventral ; thalamus Vlo/VA/MD : noyaux ventro-latéraux/ventral-anterieur/noyaux médio-dorso.

A.II.2) Les symptômes non moteur :

A.II.2.a) Dysfonction du système nerveux autonome :

Les dérégulations des systèmes cholinergiques parasympathique et sympathique et du système noradrénergique sympathique, peuvent être à l'origine de dysfonctions du système nerveux autonome. Suivant le système impliqué on observe différents symptômes pouvant parfois apparaître avant même la phase prémotrice. L'altération du système cholinergique parasympathique entraîne des problèmes aux niveaux gastro-intestinal, urogénital et pupillaire. Celle du système cholinergique sympathique provoque des dérégulations de la thermorégulation, ainsi qu'une hypo ou une hyper-transpiration. Et enfin, celle du système noradrénergique sympathique induit des dysfonctions cardiaques, une hypotension orthostatique, de même que des insuffisances du baroréflexe.

A.II.2.b) Désordres du sommeil :

Ce n'est que très récemment que les troubles du sommeil sont diagnostiqués et traités. Ces troubles se manifestent différemment selon les patients. Ainsi, les principaux désordres que l'on observe sont un sommeil fragmenté, des insomnies, une somnolence très importante qui favorisent les « attaques du sommeil » ainsi que des troubles du comportement du sommeil (mouvement rapides des yeux) (Fantini et al., 2005).

A.II.2.c) Désordres neuropsychiques :

L'apparition des désordres psychiques semble liée à l'âge et au caractère invasif de la maladie. En effet, ces symptômes semblent se déclarer lorsque les

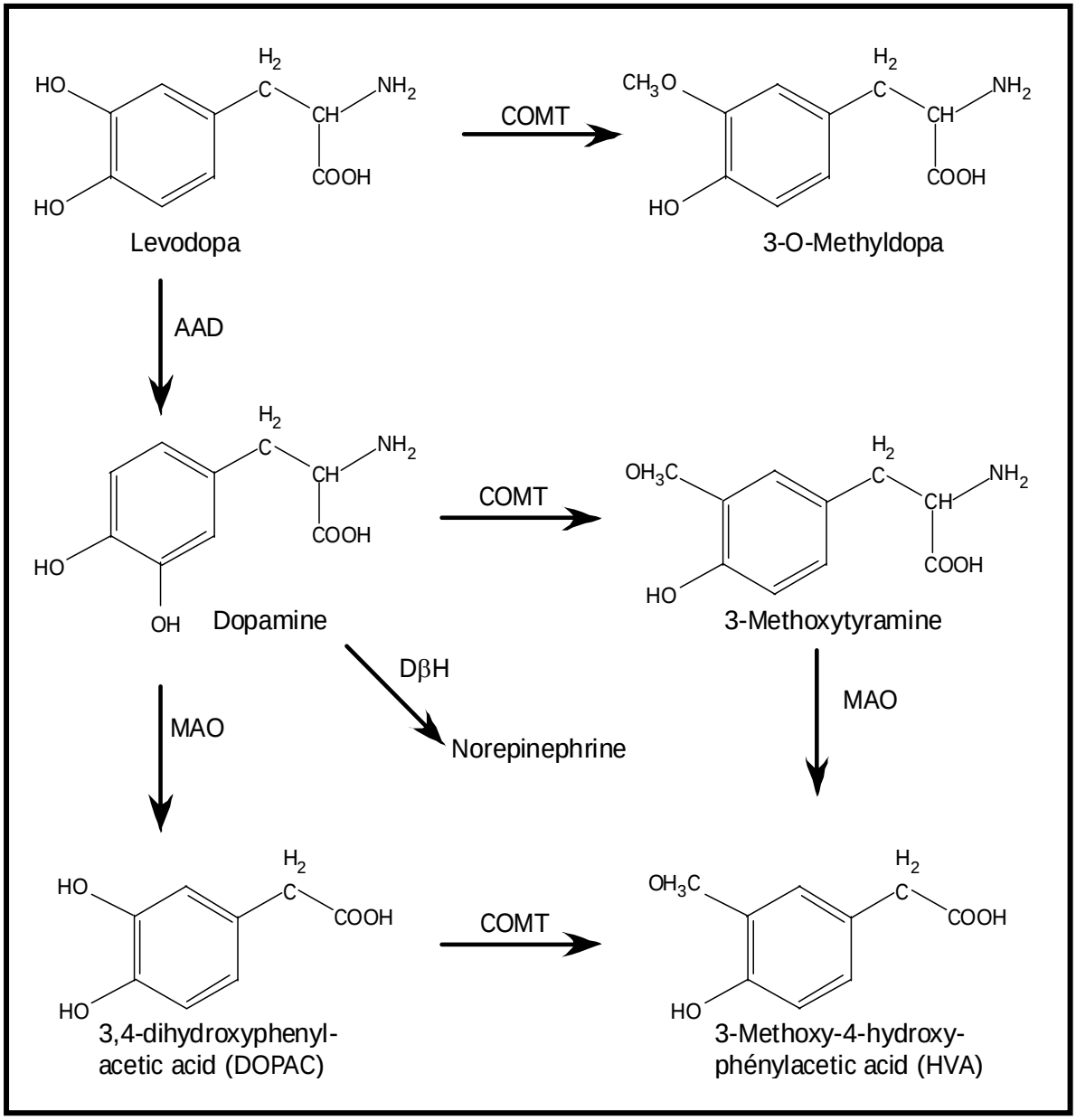
systèmes noradrénergique et sérotoninergique sont atteints. Les principaux symptômes sont la dépression et l'apathie, l'anxiété accompagnée de crises de panique (ou hyperémotivité) (Montgomery et al., 1999). Les patients peuvent avoir un comportement impulsif (syndrome lié à la dérégulation du taux de dopamine), voir compulsif (le jeu, les achats, la nourriture ou encore la sexualité) (Dubois and Pillon, 1997; Evans et al., 2004). Les malades souffrent également d'hallucinations visuelles, de confusion mentale et de psychoses (Aarsland et al., 2007).

A.III.2.d) Autres désordres :

- La majorité (80 à 90 %) des patients souffrent de troubles olfactifs comme par exemple d'hyposmie : perte des aptitudes de détection, de discrimination, et d'identification des odeurs. Cette perte des capacités olfactives apparaît avant même les premiers symptômes moteurs. L'âge d'apparition de ce symptôme, associé à sa prévalence, en font un critère important dans l'établissement du diagnostique de maladie de Parkinson (Tissingh et al., 2001).

- Les désordres neuropsychiques ainsi que les troubles du sommeil induisent un état de fatigue générale, qui se confond souvent avec l'apathie (Friedman et al., 2006).

- La synucléinopathie atteint également le système nociceptif en particulier : le noyau parabrachial, la substance grise périaqueducale, ainsi que les régions thalamiques médianes, ce qui entraîne chez les patients des douleurs musculaires (raideur, spasmes, crampes), mais aussi des douleurs articulaires (rhumatismes) (Ford, 1998; Giuffrida et al., 2005).



D'après http://www.ualberta.ca/~csps/JPPS5(2)/C.Okereke/levodopa.htm

Figure 9 : Métabolisme de la dopamine

Cette figure regroupe les enzymes ainsi que les produits issus du métabolisme de la dopamine. On peut voire par exemple que la levodopa est convertie en dopamine par une seule enzyme, la dopa-décarboxylase.

COMT : Catéchol-O-Méthyl transférase ; MAO : monoamine oxydase ; AAD : dopa-décarboxylase ; DβH : dopamine-bêta-hydroxylase.

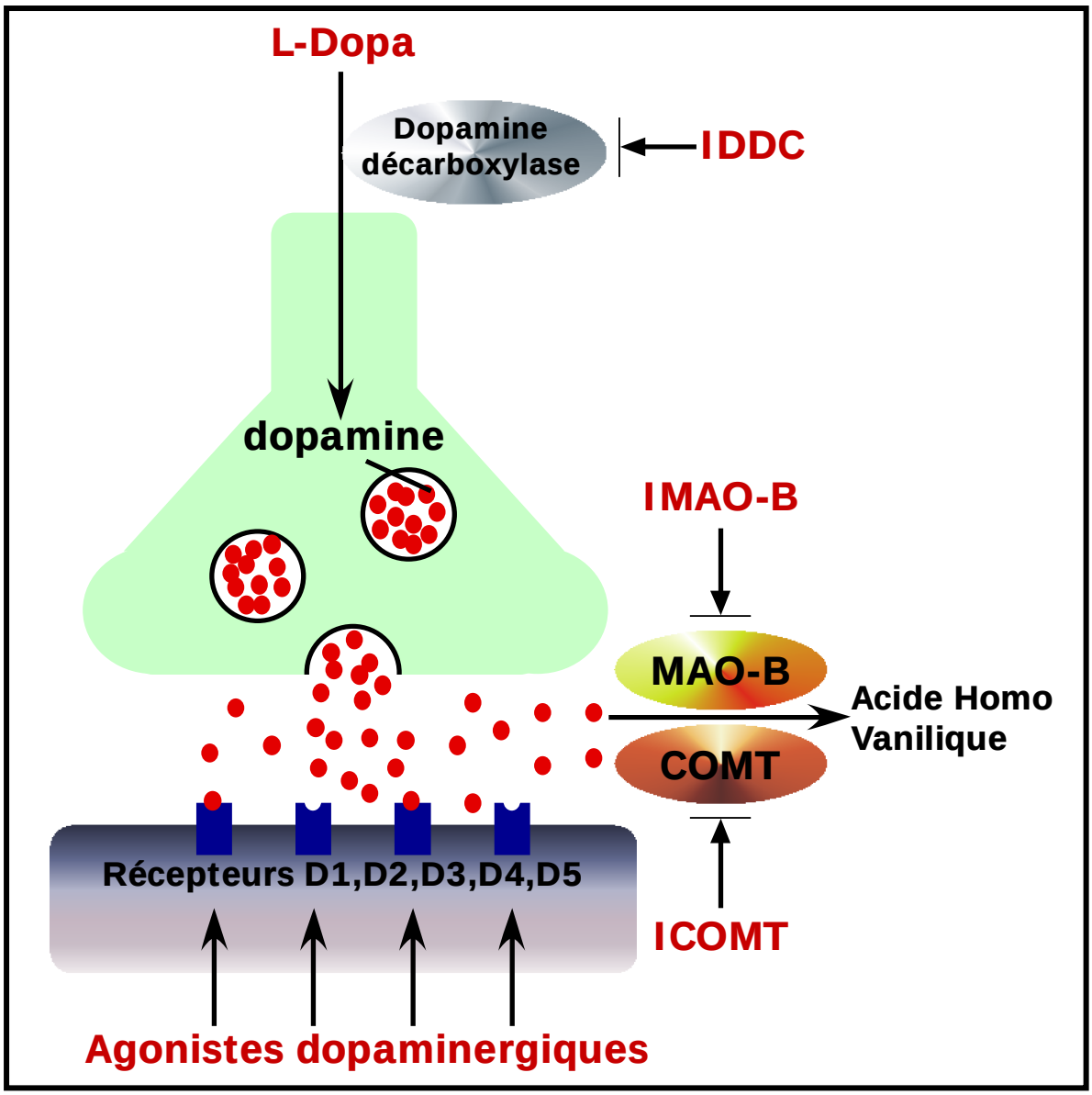
A.III) Les traitements :

Actuellement tous les traitements sont palliatifs et seuls les troubles moteurs (tremblement, bradykinésie/akinésie et rigidité) sont accessibles aux thérapies.

A.III.1) Les traitements pharmacologiques :

A.III.1.a) La L-Dopa ou levodopa:

La L-Dopa, le précurseur naturel de la dopamine, constitue le principal traitement de la maladie de Parkinson et elle est capable de traverser la barrière hémato-encéphalique. Elle se distribue dans tout l'organisme. Pour donner de la dopamine, la L-Dopa doit subir une décarboxylation, ce qui va aussi permettre son stockage au niveau des neurones. La décarboxylation au niveau périphérique entraîne des effets indésirables (hypotension artérielle, hallucinations, nausées et vomissements), en effet la dopamine n'est plus capable de franchir la barrière hémato-encéphalique (Figure 9). On associe donc la L-Dopa à d'autres molécules, soit à des molécules contrecarrant directement les effets secondaires (anti-vomitifs), soit à des inhibiteurs de la décarboxylase périphérique, permettant de diminuer les effets secondaires et d'augmenter considérablement la biodisponibilité de la L-Dopa. Lorsque la perte neuronale devient massive la L-Dopa perd son efficacité. En effet, la dopamine produite à partir de la L-Dopa est stockée par les neurones survivants. Lorsque leur nombre devient insuffisant l'état moteur des patients suit directement l'évolution de la concentration plasmatique en L-Dopa, ce qui induit l'apparition de nouveaux troubles moteurs (dyskinésies, fluctuations motrices) et une modification de la sensibilité des récepteurs dopaminergiques (Fabbrini et al., 1988). Les mesures permettant d'augmenter la demi-vie et la quantité de L-Dopa ne suffisent plus. Les



D'après http://www.med.univ-rennes1.fr/etud/pharmaco/parkinson.htm

Figure 10 : Représentation schématiques des dites d'action des principaux médicaments anti-parkinsoniens

IDDC : inhibiteur de la DOPA-décarboxylase, IMAO-B : inhibiteurs de la monoamine oxydase B, ICOMT : inhibiteurs de la catéchol-O-méthyl transférase.

classes de médicaments développés à l'heure actuelle visent à éviter les effets indésirables de la dopa-thérapie et à augmenter sa durée d'action au niveau synaptique.

A.III.1b) Agonistes dopaminergiques :

Les agonistes dopaminergiques sont des molécules qui vont directement agir sur les récepteurs dopaminergiques situés au niveau du striatum. Leur mode d'action leur permet d'être efficace même lorsque le taux de neurones dopaminergiques est faible puisqu'ils agissent directement sur les récepteurs post-synaptiques (Playford and Brooks, 1992), qui sont en partie préservés au cours de la maladie. Les agonistes dopaminergiques sont liposolubles ce qui augmente leur durée d'action mais aussi leurs liaisons aux protéines plasmatiques. Ils entraînent cependant des effets secondaires semblables à ceux induits par la L-Dopa, tels que la confusion, les hallucinations ou l'hypotension. En effet, ces agonistes stimuleraient également les systèmes cortico-limbique et sérotoninergique. Les agonistes dopaminergiques sont mieux supportés par les patients jeunes ne souffrant pas encore de troubles psychiques. Ils permettent de retarder le traitement à la L-Dopa et donc la survenue des complications motrices (Figure 10).

A.III.1.c) Les inhibiteurs enzymatiques :

Les inhibiteurs enzymatiques sont des molécules qui ont pour but de prolonger l'effet thérapeutique de la L-Dopa, pour cela ils ciblent les enzymes de dégradation de la dopamine augmentant ainsi sa demi-vie et sa biodisponibilité. Leur cibles sont la monoamine oxydase B (MAO-B), qui dégrade la dopamine au niveau du cerveau, et la Catéchol-O-Méthyl Transférase (COMT), qui transforme la L-DOPA au niveau périphérique, en 3-O-méthyldopa (3-OMD), un métabolite inactif présumé antagoniser la pénétration de la L-DOPA dans le cerveau (Figure 9 et 10).

A.III.1.d) Les anti-cholinergiques :

Les anti-cholinergiques ont pour effet de réduire l'hyperactivité cholinergique striatale induite par la réduction du tonus inhibiteur dopaminergique. Ils ont principalement une action sur les tremblements (Fahn, 1998), mais sont également efficaces contre l'hypersalivation. Ces molécules sont essentiellement dérivées de l'atropine. Elles possèdent de nombreux effets secondaires périphériques indésirables (sécheresse buccale, troubles de l'accommodation, constipation...), mais aussi centraux (confusion, troubles mnésiques).

Les traitements pharmacologiques sont généralement accompagnés d'une rééducation (kinésithérapie, orthophonie, ergothérapie) qui doit être débutée précocement.

A.III.2) La chirurgie fonctionnelle :

Récemment, une nouvelle approche a été envisagée afin de soulager les patients : la chirurgie fonctionnelle. Elle découle de recherches effectuées sur la stimulation électrique de différentes zones du cerveau. Avec le développement des techniques de stimulation profonde, un protocole chirurgical a pu être mis en place dans le cadre de la maladie de Parkinson (Benabid et al., 2000). Cette technique concerne 10% à 15% des patients répondant à des critères bien précis : 1. L'opération concerne les formes les plus sévères ne répondant plus au traitement à la L-Dopa. 2. Les patients ne doivent pas souffrir de désordres neuropsychique car cette technique peut parfois entraîner des troubles de l'humeur (dépression, anxiété) et donc fortement accentuer ces désordres. Les risques liés à toute intervention chirurgicale s'ajoutent aux critères neuropsychiques empêchant les patients de plus de 70 ans de

subir cette intervention. L'opération consiste en l'introduction d'électrodes de stimulation au niveau des ganglions de la base. La stimulation électrique constante va permettre aux différents noyaux de retrouver une activité normale en les désynchronisant (Perlmutter and Mink, 2006). Cette technique est efficace pour corriger les tremblements, l'akinésie et la raideur, mais est moins efficace sur les troubles de l'équilibre. La chirurgie fonctionnelle chez la majorité des patients opérés est très efficace mais reste un traitement palliatif ne stoppant pas l'évolution de la maladie.

A.III.3) Quel avenir ?

Actuellement les nombreuses voies de recherche sont axées sur l'arrêt de l'évolution de la maladie ou le remplacement des neurones dopaminergiques. Il existe trois grands axes thérapeutiques.

- La thérapie génique consiste à injecter un virus modifié (adénovirus) portant des gènes d'intérêt. Par exemple, le gène de la décarboxylase de l'acide glutamique (GAD), enzyme clé de la biosynthèse du GABA (acide gamma-amino butyrique, principal neurotransmetteur inhibiteur du système nerveux), dans le noyau sous-thalamique afin d'inhiber son activité (Kaplitt et al., 2007).

- L'utilisation de facteurs neurotrophiques est une approche qui vise à protéger ou régénérer les neurones dopaminergiques par l'action ciblée de protéines endogènes régulant la survie, la différenciation, sur des sites cérébraux spécifiques, par exemple en utilisant le facteur neurotrophique dérivé des cellules gliales (GDNF) (Dass et al., 2006).

- La greffe de neurones est également à l'essai. Ainsi, les greffes de neurones dopaminergiques embryonnaires (posant un problème éthique) ou de neurones issus

du cortex ou de la moelle du patient cultivée *in vitro* puis réinjectés (Lindvall and Kokaia, 2006), sont à l'étude.

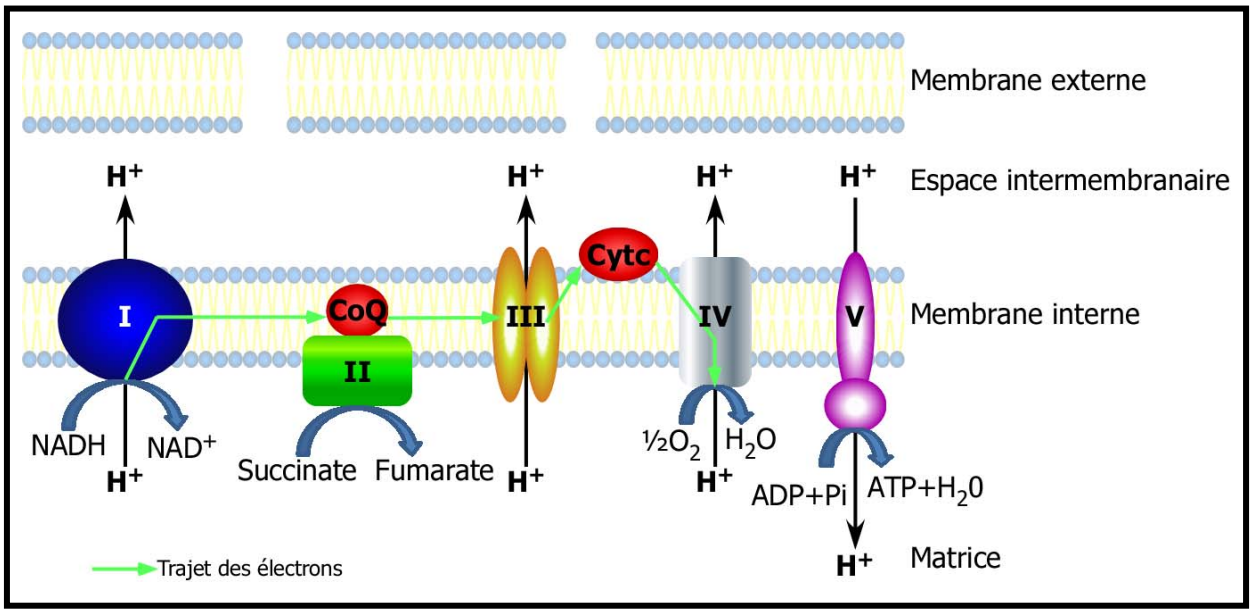


Figure 11 : Représentation schématique de la chaîne respiratoire de la mitochondrie

La chaîne respiratoire (aussi appelée phosphorylation oxydative) est constituée d'un ensemble de complexes protéiques qui servent à ré-oxyder les coenzymes NADH et FADH2 qui ont été réduits au cours du cycle de Krebs. Cette ré-oxydation s'accompagne de la création d'un gradient transmembranaire de protons. Le gradient de proton va servir à fabriquer de l'ATP, molécule énergétique.

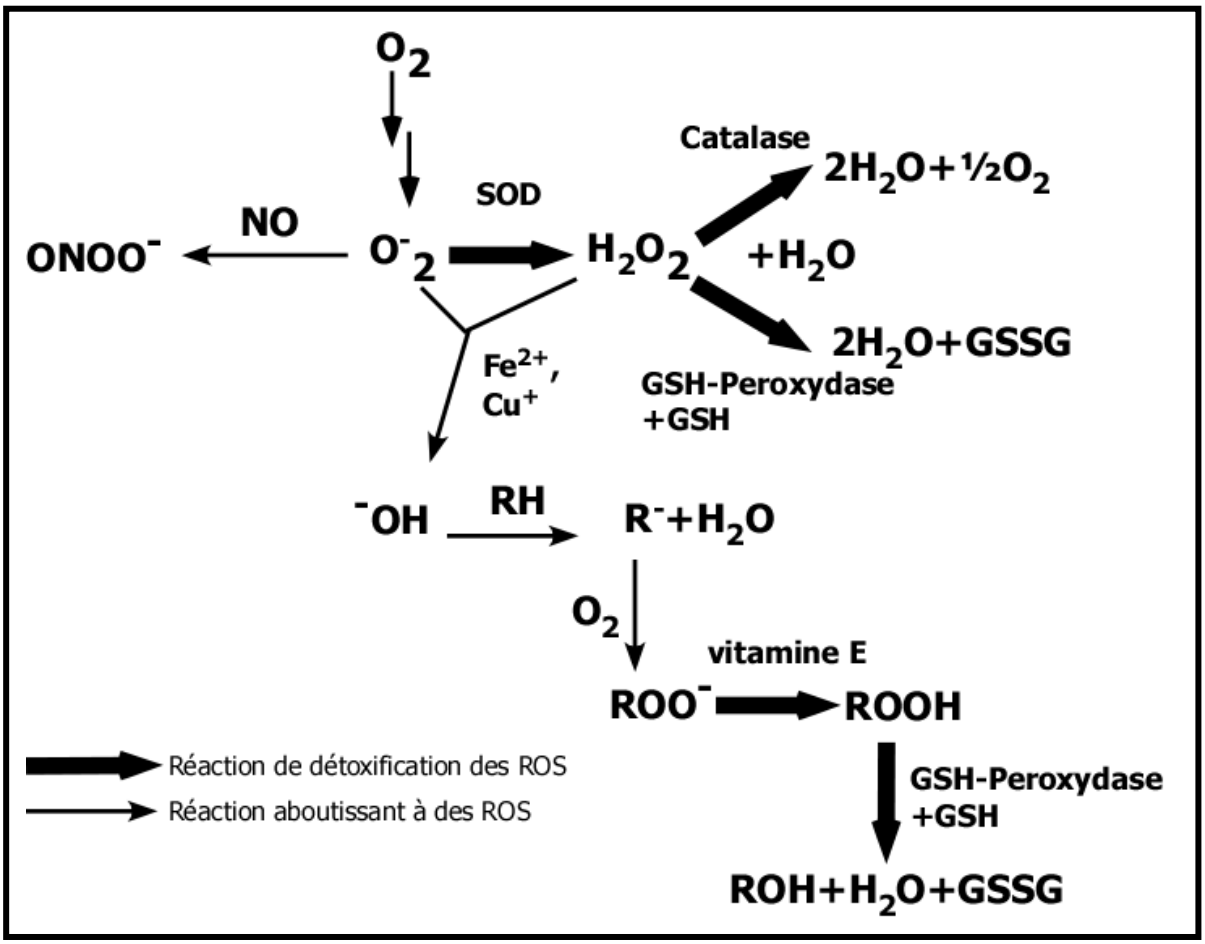
I : complexe I, NADH déshydrogénase ; II : complexe II, succinate déshydrogénase ; III : complexe III, Ubiquinol-cytochrome c oxydoréductase : IV : complexe IV, cytochrome c oxydase ; V : complexe V, ATP synthase ; CoQ : coenzyme Q ou ubiquinol ; Cytc : cytochrome c.

A.IV) L'étiologie de la maladie de Parkinson :

La pathogénèse de la maladie de Parkinson est fréquemment associée à trois dysfonctions majeures qui apparaissent dans les cerveaux des patients. Des dysfonctions de la mitochondrie associées au stress oxydatif sont considérées comme jouant un rôle majeur dans le développement de cette maladie, à ceux-ci s'ajoute une augmentation des protéines mal repliées provoquée par des défauts au niveau du système ubiquitine-protéasome. Que ce soit dans les cas de formes sporadiques ou de formes familiales, des dysfonctions au niveau de ces voies entraînent une dégénérescence neuronale.

A.IV.1) Dysfonction mitochondriale :

Le lien direct entre la maladie de Parkinson et des dysfonctions mitochondriales a été établi par une étude post-mortem, décrivant une déficience du complexe-I de la chaîne respiratoire (Figure 11) au niveau de la substance noire des patients dans les neurones et dans la glie (Mizuno et al., 1989; Schapira et al., 1989). Cette déficience du complexe-I est également visible au niveau du muscle squelettique et des plaquettes (Bindoff et al., 1989; Parker et al., 1989). Dans la maladie la Parkinson seule la stabilité structurale et l'activité du complexe-I de la chaîne respiratoire est affecté par une augmentation de l'oxydation endogène (Keeney et al., 2006). Ces défaillances au niveau du complexe-I entrainent une augmentation des processus apoptotiques dépendants de la mitochondrie liée à une diminution de la production d'ATP, de la génération de radicaux libres, et à une sensibilisation accrue des cellules à la protéine pro-apoptotique Bax (Perier et al., 2005). Ces défauts au niveau de la mitochondrie pourraient avoir des causes



http://fr.wikipedia.org/wiki/Radical_(chimie)

Figure 12 : Réaction aboutissant à la formation ou à la détoxification des ROS

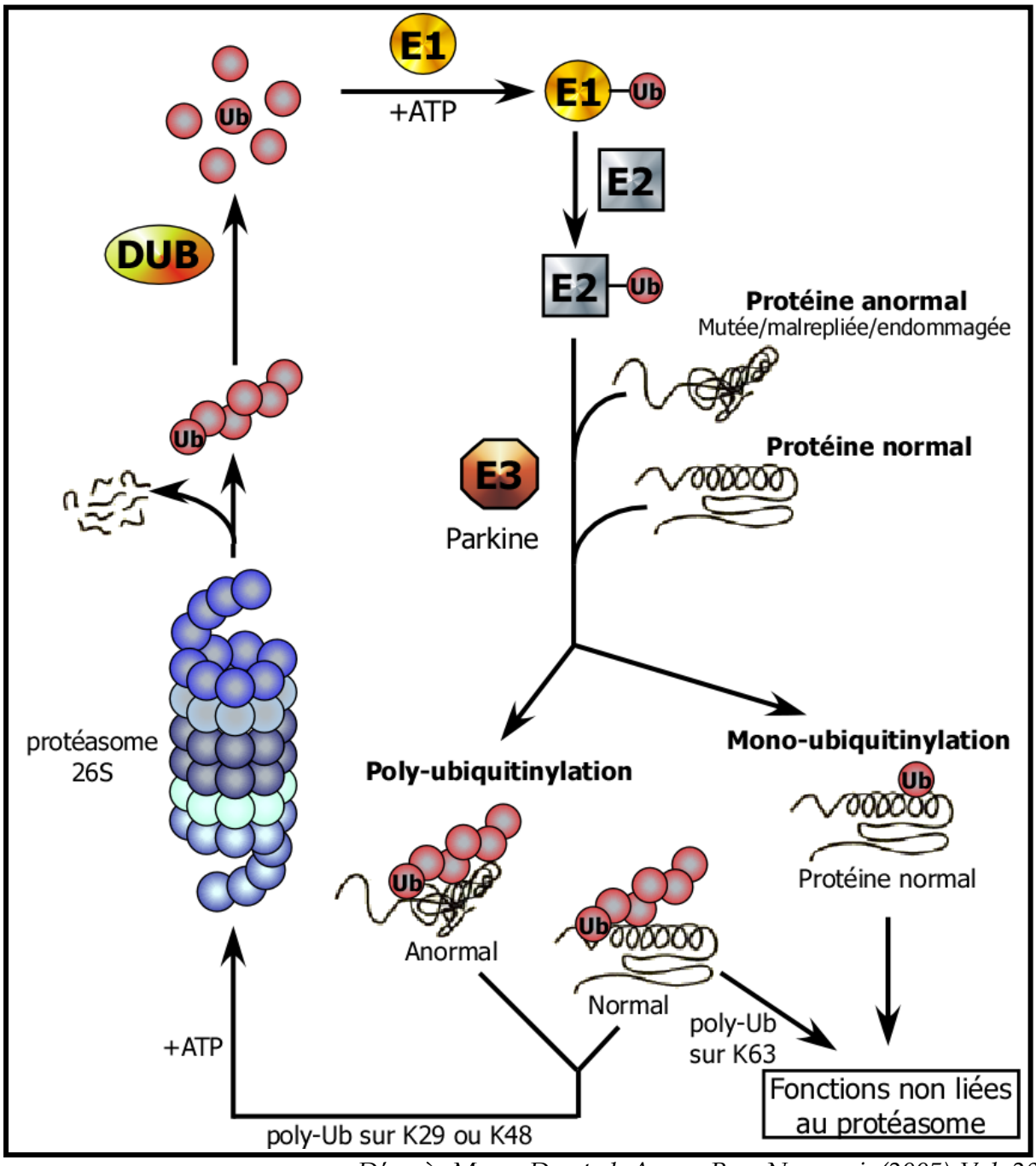
SOD : superoxyde dismutase ; GSH-peroxydase : glutathion peroxydase ; O_2^- : radical superoxyde ; H_2O_2 : peroxyde d'hydrogène ; HO^-°: radical hydroxyle ; NO : monoxyde d'azote ; O_2 : dioxygène ; $ONOO^-$: l'ion peroxynitrite ; ROO^- : radical peroxyle ; RO^- : radical alkoxyle (R : chaîne carbonée).

génétiques ou environnementales, que je détaillerai dans le chapitre consacré aux origines de la maladie.

A.IV.2) Stress oxydatif :

Le stress oxydatif (ou stress oxydant) est un stress induit par des espèces réactives oxygénées (ROS, « Reactive Oxygen Species ») et par des espèces réactives oxygénées et azotées (RONS, « Reactive Oxygen Nitrogen Species »). Ces espèces sont des radicaux libres qui comprennent par exemple le peroxyde d'hydrogène (H_2O_2) qui, en présence de fer (sous forme ionique), donne deux radicaux hydroxyle (•OH), le monoxyde d'azote (NO•) ou encore les radicaux dérivant d'acides gras insaturés (Figure 12). Il existe un mécanisme permettant la détoxification de ces ROS, mais lorsque le système n'arrive plus à détoxyfier (lorsqu'il est dépassé) on a un stress oxydant (Figure 12).

Des études post-mortem ont clairement montré l'implication des dommages provoqués par les radicaux libres dans la pathogénèse de la maladie de Parkinson, en particulier des détériorations au niveau des protéines, des lipides et de l'ADN sont détectées au niveau de la substance noire de patients (Jenner, 2003). Le stress oxydatif est considéré comme compromettant l'intégrité des neurones vulnérables, contribuant ainsi à la dégénérescence neuronale. Les causes de l'augmentation du stress oxydatif restent inconnues, cependant l'accroissement du taux de radicaux libres pourrait être expliqué par les dysfonctions mitochondriales, l'augmentation du métabolisme de la dopamine produisant en excès du peroxyde d'hydrogène et d'autres ROS, l'accroissement du taux d'oxyde ferreux et une diminution de l'efficacité des voies de défenses anti-oxydantes (Jenner, 2003). En effet, l'inhibition ou une mutation au niveau du complexe-I de la chaîne respiratoire induisent une augmentation de la production de radicaux libres. Inversement une augmentation du stress oxydatif induit des dysfonctions mitochondriales (un environnement oxydatif



D'après Moore D., et al, Annu. Rev. Neurosci. (2005) Vol. 28

Figure 13 : Représentation schématique du système ubiquitine-protéasome

L'ubiquitinylation des protéines peut conduire soit à leurs dégradations par le système ubiquitine-protéasome soit à leurs activations.

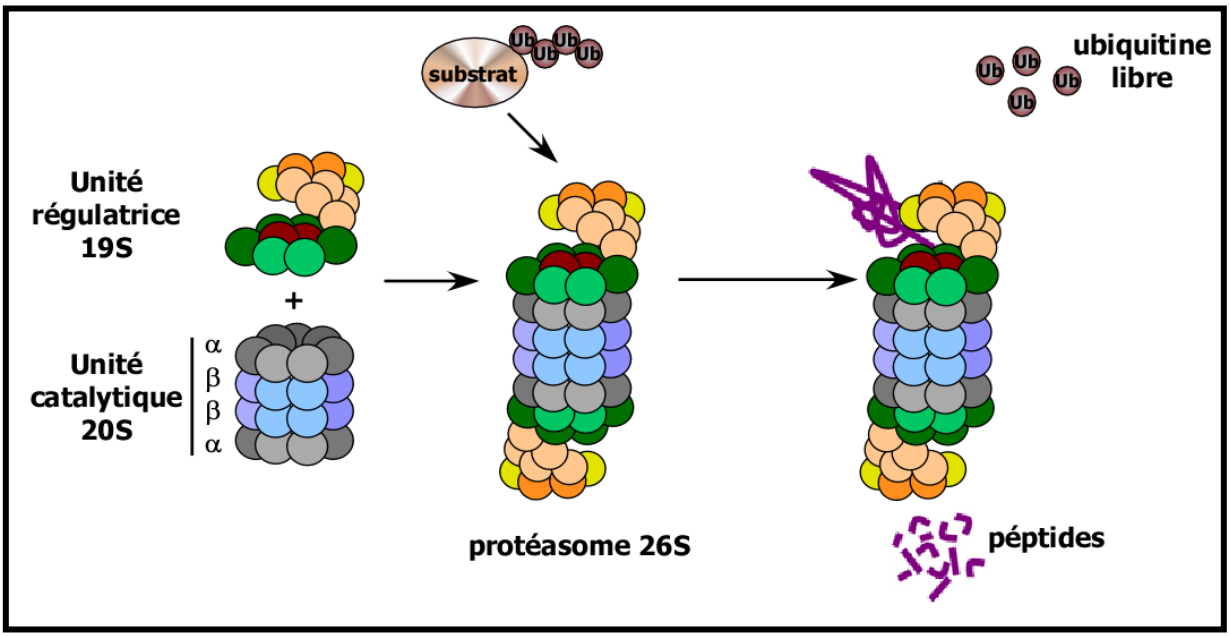
Ub : ubiquitine, DUB : enzyme de dé-ubiquitinylation ; E1 : enzyme activant l'ubiquitine ; E2 : enzyme conjuguant l'ubiquitine ; E3 : ubiquitine ligase

et la phosphorylation oxydative sont associés à la production de ROS) (Schapira, 1995; Thomas et al., 1993). Le stress oxydatif et les dysfonctions mitochondriales sont deux mécanismes étroitement liés, en effet, l'un dépend en partie de l'autre et vice versa.

A.IV.3) Altérations du système ubiquitine- protéasome :

Le système ubiquitine-protéasome est un système de dégradation qui est majoritairement responsable du renouvellement protéique (Hershko and Ciechanover, 1998). Les protéines destinées à la dégradation sont marquées sur un résidu lysine (majoritairement le K68) avec une molécule d'ubiquitine (une protéine de 76 aminoacides) qui se fixe par son résidu G76 (en C-terminal). Cette réaction de fixation requiert une série élaborée d'actions séquentielles de l'enzyme activant l'ubiquitine (E1), puis de l'enzyme conjuguant l'ubiquitine (E2), et enfin de l'ubiquitine ligase (E3) (Hershko and Ciechanover, 1998) (Figure 13). Habituellement, ce processus impliquant plusieurs enzymes est répété plusieurs fois pour former une chaîne polyubiquitinylée sur le substrat. Le substrat polyubiquitinylé est dirigé vers le protéasome 26S, un large complexe protéique comprenant un corps protéolytique 20S, et deux unités régulatrices 19S. La sous-unité 20S contient quatre structures heptamériques formées par des sous-unités α et β, cette sous-unité 20S possède différentes activités peptidasiques de type trypsine, chymotrypsine, caspase (Stein et al., 1996). Les sous-unités régulatrices 19S jouent un rôle dans l'initiation de la protéolyse : reconnaissance, débobinage, et translocation des substrats dans le corps protéolytique (Braun et al., 1999; Glickman et al., 1998; Navon and Goldberg, 2001) (Figure 14). A la suite de la dégradation, il y régénération des monomères d'ubiquitine par l'action d'enzyme de dé-ubiquitinylation (DUBs).

L'altération de l'activité du protéasome ainsi que le mauvais repliement des protéines apparaissent comme impliqués dans la pathogénèse aussi bien des formes



D'après Marteijn J., et al, Leukemia (2006) vol. 20

Figure 14 : Représentation schématique des différentes sous-unités du protéasome 26S

Le protéasome 26S se compose d'une unité catalytique, de coefficient de sédimentation 20S contenant quatre structures heptamériques formées par deux sous-unités différentes (α et β), qui est associée à deux sous-unités régulatrices19S sur chacune de ses exterminés.

Ub : ubiquitine

familiales que des formes sporadiques de la maladie de Parkinson (Giasson and Lee, 2003; Moore et al., 2003a). En accord avec cette affirmation, dans la substance noire des patients parkinsoniens on trouve des déficits structuraux (perte des sous-unités α du 20S) et fonctionnels (perte d'activité protéolytique) au niveau du protéasome 26S (McNaught et al., 2003; McNaught et al., 2002). Des rats exposés de façon systémique à des inhibiteurs naturels ou synthétiques du protéasome, présentent des symptômes de la maladie de Parkinson : parkinsonisme progressif, neurodégénérescence spécifique de la voie nigro-striatale et la formation d'inclusions intracytoplasmiques semblables aux corps de Lewy, contenant de l'α-synucléine et de l'ubiquitine (McNaught et al., 2004). Ceci suggère que les dysfonctions du protéasome sont le point commun final conduisant à la dégénérescence des neurones dopaminergiques. De plus, la parkine une ubiquitine ligase et l'UCHL-1 impliquées dans les formes familiales de la maladie font partie du système ubiquitine-protéasome ce qui renforce l'idée de l'implication de celui-ci dans la pathogenèse (Leroy et al., 1998; Shimura et al., 2000; Zhang et al., 2000).

Les dysfonctions mitochondriales, le stress oxydatif et les altérations du système ubiquitine-protéasome sont donc des voies interconnectées importantes dans la pathogénèse des formes familiales et sporadiques de la maladie de Parkinson qui conduisent à la mort des neurones dopaminergiques.

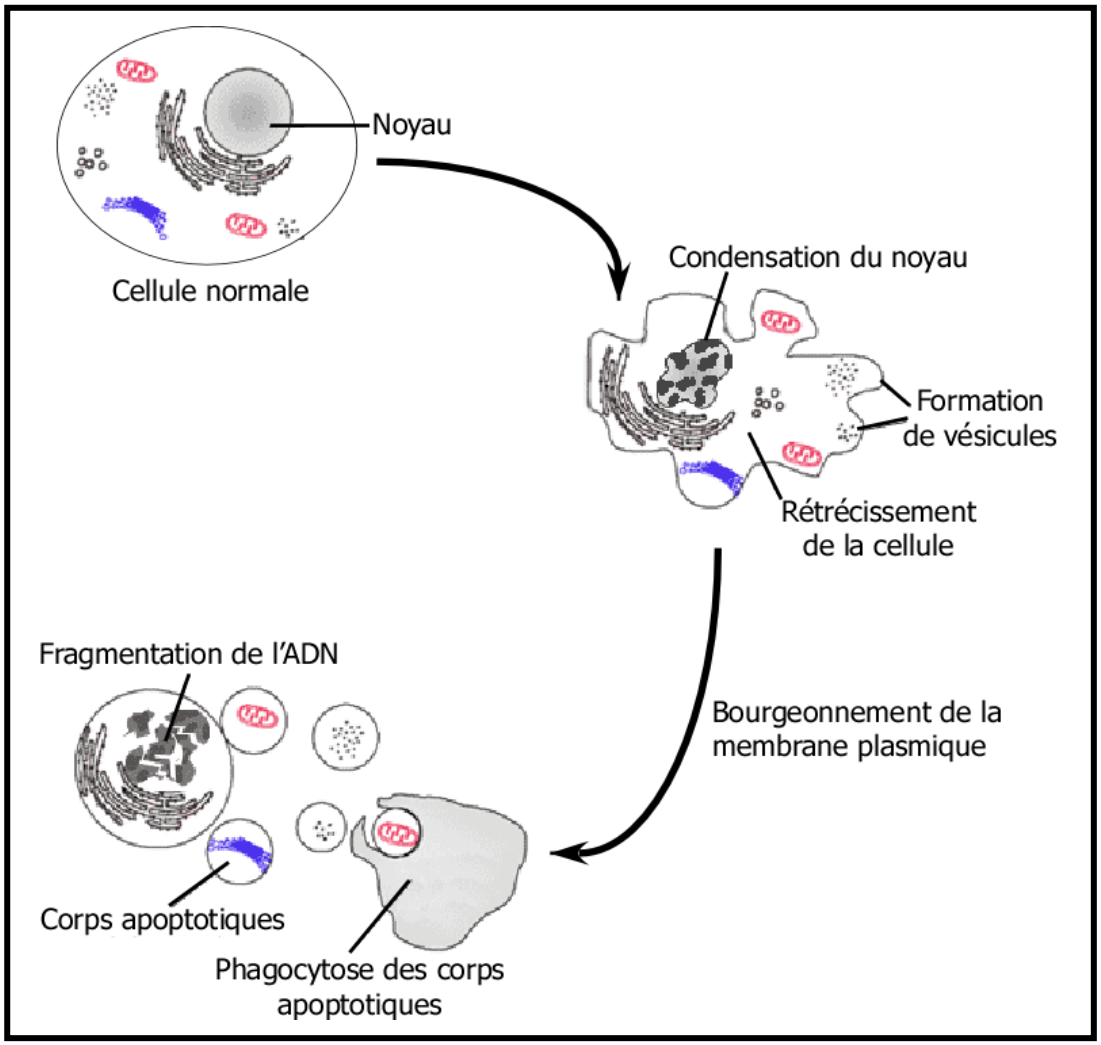


Figure 15 : Evolution morphologique d'une cellule en apoptose

Une cellule en apoptose va subir toute une série de changements microscopiques, macroscopiques et morphologiques qui vont aboutir à sa phagocytose par les macrophages sans jamais compromettre l'intégrité de sa membrane plasmique.

A.V) Mort cellulaire par apoptose et maladie de Parkinson :

A.V.1) Généralités sur l'apoptose :

L'apoptose est une mort cellulaire génétiquement programmée, hautement régulée et déclenchée en réponse à des signaux cellulaires lors de nombreux processus physiologiques où elle est en équilibre constant avec la prolifération cellulaire. Elle contribue, par exemple, au cours de l'embryogénèse, au modelage du cerveau, à la disparition de la palmure des mains ou encore à la disparition de l'appendice caudale au niveau du dos. L'apoptose permet également d'éliminer des cellules devenues inutiles, ou endommagées par un stress induisant des dommages à l'ADN (irradiation aux UV ou aux rayons X), ou par l'agrégation de protéines ayant des problèmes de conformation.

Dans un laps de temps assez court (quelques heures), la cellule en apoptose va subir de nombreux changements morphologiques et intracellulaires qui n'altéreront pas l'intégrité de la membrane plasmique, ce qui empêchera le déversement du contenu cellulaire et limitera donc l'inflammation, contrairement à la nécrose qui est une mort accidentelle induisant une forte réaction inflammatoire. L'apoptose commence par une perte d'adhésion de la cellule qui s'isole, suivie par une réduction du volume cellulaire suite à la condensation du noyau et du cytoplasme. La mitochondrie va subir des modifications majeures : relargage du cytochrome c dans le cytoplasme, diminution du potentiel de membrane et ouverture de pores. S'en suivent, une fragmentation de l'ADN et un bourgeonnement de la membrane plasmique qui conduisent à la formation de vésicules : les corps apoptotiques (Figure 15). Lors du bourgeonnement des corps apoptotiques il y a externalisation des molécules de phosphatidylsérine localisées normalement dans le feuillet interne de la

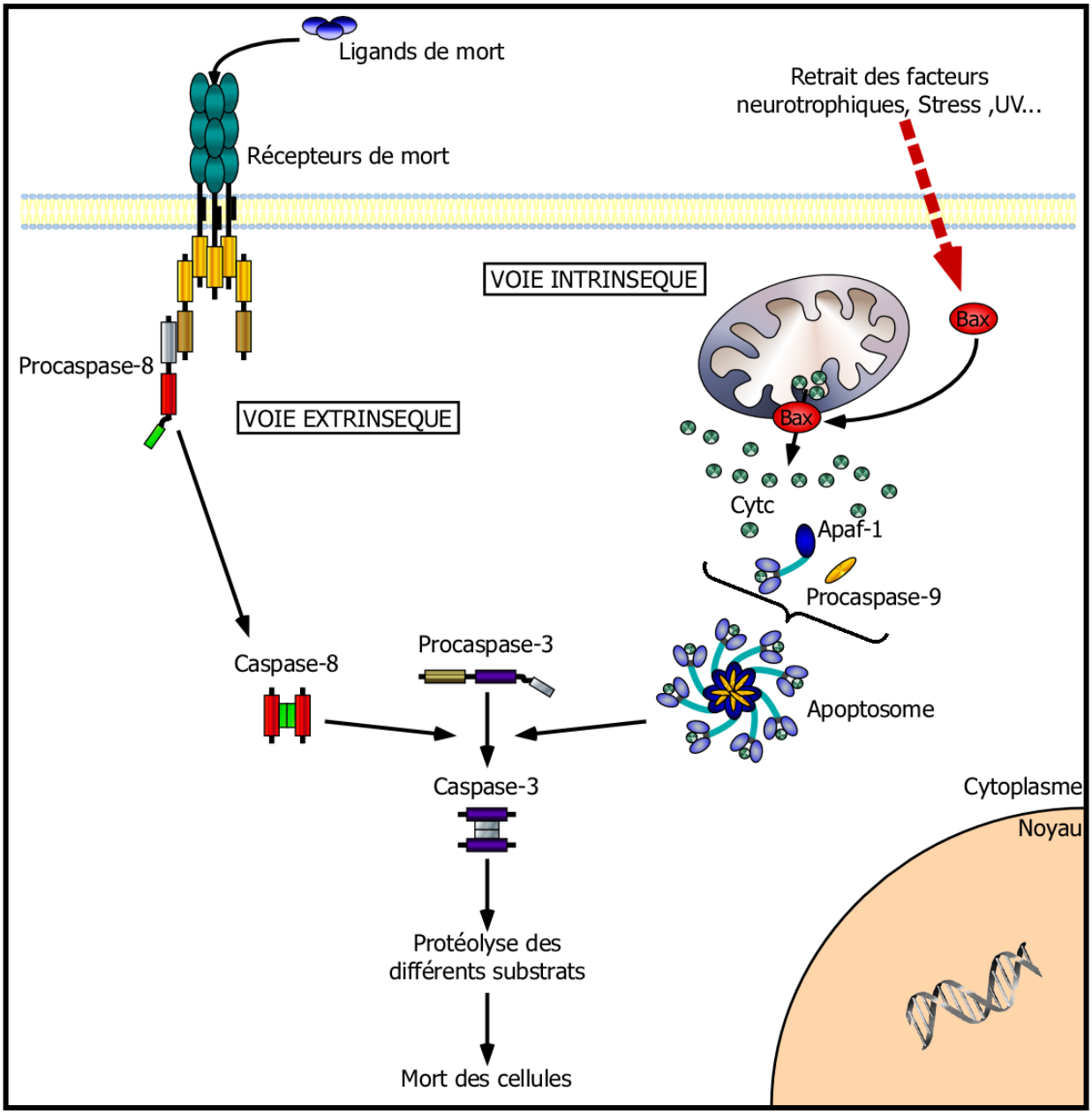


Figure 16 : Représentation simplifiée des voies intrinsèque et extrinsèque

Suivant le type de stimuli la voie apoptotique empruntée sera différente mais aboutira à l'activation des caspases effectrices. L'une de ces voies est dite extrinsèque et fait intervenir des récepteurs transmembranaires. L'autre est dite intrinsèque est implique la mitochondrie ainsi que de nombreux intermédiaires qui ne sont pas représentés sur ce schéma.

membrane plasmique, ce qui permet leur reconnaissance et leur phagocytose par les macrophages.

Il existe trois voies principales qui conduisent à l'initiation des événements moléculaires provoquant une mort cellulaire programmée : la voie intrinsèque, la voie extrinsèque (Figure 16) et la voie du stress réticulaire que je n'aborderai pas ici (Burke, 2008). L'apoptose par la voie extrinsèque est initiée lors de la fixation de ligands sur les récepteurs de mort de la superfamille des TNFs (« tumor necrosis factor »). La voie intrinsèque est induite par différents signaux cellulaires et passe par une cascade moléculaire au niveau de la mitochondrie, impliquant les membres de la superfamille Bcl-2, aboutissant au relargage du cytochrome c dans le cytoplasme. Ces deux voies tendent à l'activation des caspases effectrices et en particulier la caspase-3. Les caspases sont des protéases à cystéine, très conservées au cours de l'évolution et ayant différentes fonctions. On trouve, en effet, les caspases initiatrices (2, 8, 9 et 10), les caspases effectrices (3, 6 et 7) et les caspases intervenants dans la maturation des cytokines (1, 4, 5, 13, 14 et 11, 12 chez la souris uniquement). L'activation des caspases va induire le clivage de leurs nombreux substrats, par exemple PARP qui est une enzyme de réparation de l'ADN, induisant ainsi la mort cellulaire.

A.V.2) p53 une protéine clé impliquée dans les différentes voies apoptotiques :

Le gène de p53 est situé sur le chromosome 17p13.1, il est très conservé au cours de l'évolution et code pour une phosphoprotéine de 393 acides aminés et de poids moléculaire 53kDa. La protéine p53 est avant tout un facteur de transcription possédant de nombreux gènes cibles intervenants dans différents processus cellulaires : réparation de l'ADN, cycle cellulaire, angiogénèse et apoptose (Figure

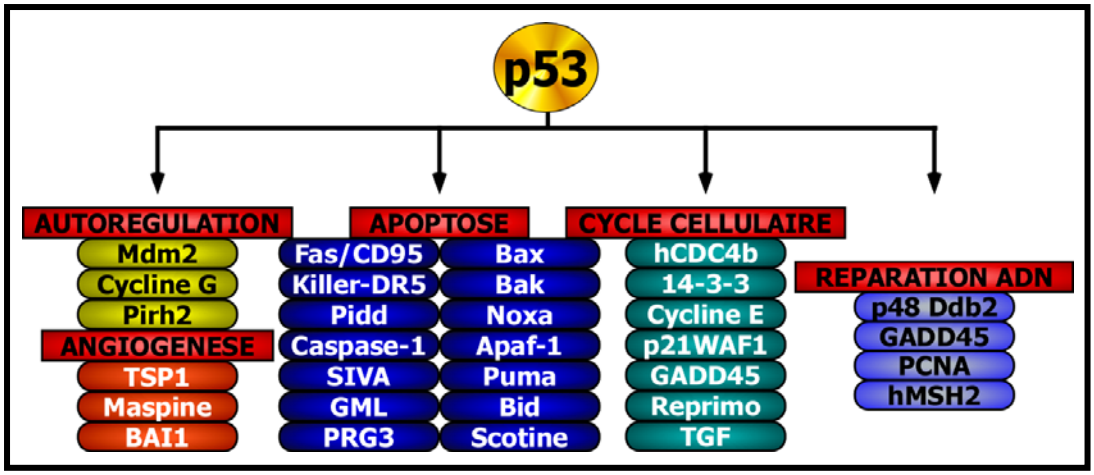
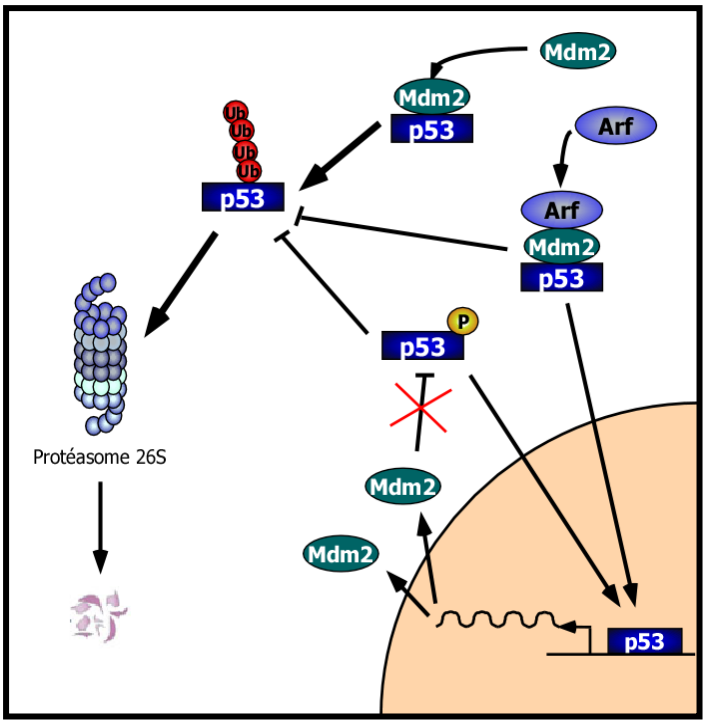


Figure 17 : Exemple de cibles transcriptionnelles de p53

Ne sont représenté ici que quelques exemples de cibles transcriptionnelles de l'oncogène p53. Sur ce schéma apparaissent les cibles impliquées dans les processus d'apoptose, de cycle cellulaire ou de réparation de l'ADN.



D'après Fåhraeus R., Nat Rev Mol Cell Biol. (2005) Vol 6

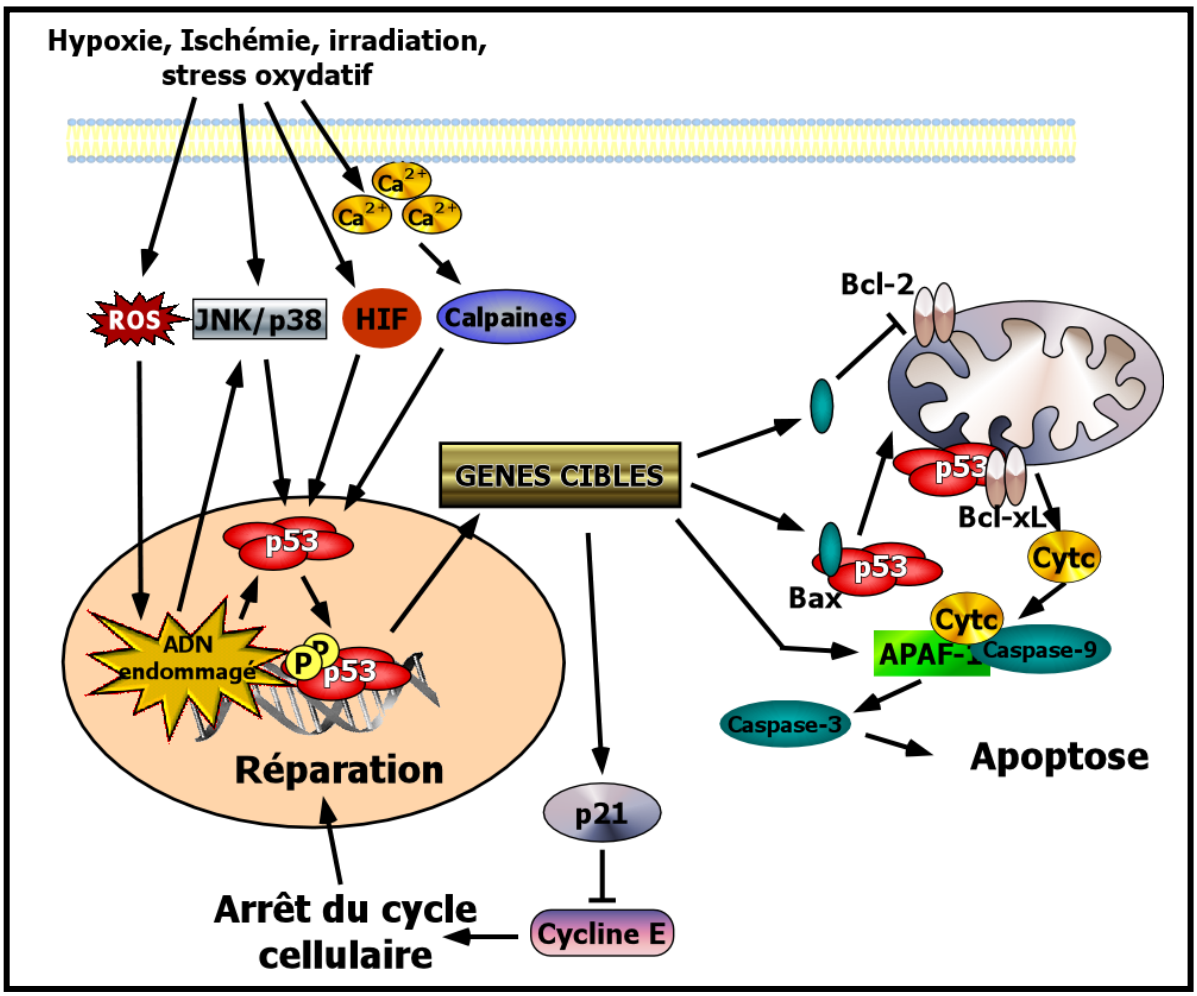
Figure 18 : Boucles de régulation positive et négative entre p53/Mdm2

Le facteur de transcription p53 transactive le promoteur de la protéine Mdm-2, cette protéine induit l'ubiquitinylation et la dégradation de p53.

17). En conditions physiologiques dans une cellule le taux de p53 est faible, sa demi-vie est très courte et elle est sous forme inactive. Le niveau de p53 est régulé très finement par la protéine Mdm2 (« murine double minute »). Il existe une boucle de régulation entre ces deux protéines : Mdm2 favorise l'ubiquitinylation et la dégradation de p53 par le protéasome et la transcription de Mdm2 est sous le contrôle de p53 (Figure 18). Lorsque la cellule est soumise à un stress : lésions de l'ADN suite à une irradiation, hypoxie ou stress oxydatif, l'association entre Mdm2 et p53 est abolie (Figure 18). Ceci entraîne une augmentation du taux de p53, son activation et sa stabilisation par des modifications post traductionnelles (phosphorylation, acétylation, sumoylation), afin de permettre la transcription de ses gènes cible. Cette séquence d'événements engendre, soit l'arrêt du cycle cellulaire, soit l'apoptose. Lorsqu'il y a arrêt du cycle cellulaire les voies de réparation sont activées par p53, le taux de p53 revient à la normale et le cycle cellulaire reprend. A l'inverse si les dommages sont trop importants, p53 va faire entrer la cellule dans un cycle apoptotique en transactivant les promoteurs de ses gènes cibles impliqués dans la cascade apoptotique (Figure 19). p53 transloque également à la membrane mitochondriale où il interagit avec les protéines de la famille Bcl-2 ce qui conduit à une modification de la perméabilité mitochondriale, suivie du relargage du cytochrome c dans le cytoplasme (Figure 19). p53 est donc une molécule indispensable au maintien de l'intégrité de la cellule et de son ADN, pour cette raison elle est également nommée « gardienne du génome ».

A.V.3) L'importance de l'apoptose et de p53 dans la maladie de Parkinson :

L'implication de l'apoptose dans la dégénérescence des neurones dopaminergiques a longtemps été un sujet de controverse mais depuis quelques années son rôle a été démontré (Hartmann et al., 2000; Hirsch et al., 1999; Hirsch et



D'après http://www.uni-marburg.de/fb16/pharmtox/gruppen/ag_culmsee

Figure 19 : Représentation simplifiée des deux voies pouvant être induites par p53 après un stress

Lors d'un stress la cellule va subir différents dommages en particulier des dommages au niveau de son ADN, p53 est activé pour induire soit, la réparation de l'ADN si cela est possible soit, l'entrée en apoptose de la cellule si les dommages sont trop importants. La voie intrinsèque est alors sollicitée.

al., 2000; Mochizuki et al., 1996; Tatton, 2000; Tatton and Kish, 1997). En effet, grâce à des expériences de TUNEL sur des cerveaux de patients parkinsoniens, Mochizuki et al. ont réussi à marquer de l'ADN fragmenté et donc à marquer des cellules en apoptose (Mochizuki et al., 1996), ceci fut confirmé par une méthode de double marquage fluorescent (Tatton et al., 1998). De plus, les formes actives des caspases-8, -9, -1 et -3 sont présentes en forte quantité dans les cerveaux de patients au niveau de la substance noire, confirmant ainsi l'implication des voies apoptotiques dans la mort des neurones dopaminergiques (Andersen, 2001; Mogi et al., 2000). Une fois l'apoptose identifiée comme étant le mécanisme impliqué dans la dégénérescence des neurones dopaminergiques, la question fut de savoir quelles étaient les voies impliquées dans le déclenchement de ce processus. Des études post-mortem sur des cerveaux de patients montrent une augmentation du niveau d'expression de la protéine Bax, mais également de GAPDH et de la caspase-3. La GAPDH (Glycéraldéhyde-3-phosphate déshydrogénase) est une protéine multifonctionnelle impliquée dans la signalisation apoptotique, et en particulier dans la voie p53-GAPDH-Bax (Berry and Boulton, 2000; Dastoor and Dreyer, 2001), ce qui suggère l'implication de la voie mitochondriale (Hartmann et al., 2001; Tatton, 2000). Cependant, d'autres études post-mortem ont également suggéré l'implication de FAS, FADD (deux composants des récepteurs de mort) et de la caspase-8, le taux de ces trois protéines étant élevé dans les cerveaux de patients parkinsoniens (Ferrer et al., 2000; Hartmann et al., 2002; Hartmann et al., 2001; Mogi et al., 1996). Tout ceci confirme l'implication des voies intrinsèque et extrinsèque dans l'initiation de l'apoptose dans les mécanismes physiopathologiques de la maladie de Parkinson.

Comme on vient de le voir le facteur de transcription p53 est également impliqué dans ces processus puisque la voie p53-GAPDH-BAX est activée *in vivo* afin d'initier l'apoptose (Tatton et al., 2003). En accord avec ces observations, des études dans les cerveaux de parkinsoniens ont montré une élévation du taux de p53 dans ces derniers quand ils sont comparés à des cerveaux contrôles (Mogi et al., 2007). L'implication de la voie apoptotique dépendante de p53 a également été démontrée dans des modèles cellulaires de maladie de Parkinson. En effet, dans des cultures primaires de neurones dopaminergiques soumises à un stress oxydatif ou à des

inhibiteurs du protéasome le taux de p53 phosphorylé est augmenté, ce qui est en accord avec des observations post-mortem (Nair, 2006; Nair et al., 2006). De plus, l'implication de ces voies a également été mise en évidence dans les modèles *in vivo* de maladie de Parkinson (Vila et al., 2008) (modèles qui seront décrits dans un chapitre leur étant consacré).

De façon intéressante, plusieurs protéines impliquées dans les formes familiales de la maladie ont un rôle dans les mécanismes apoptotiques passant par une interaction avec p53. Les travaux présentés dans ce manuscrit illustrent le rôle de plusieurs de ces protéines dans ces processus. Par ailleurs, au laboratoire, la fonction protectrice de l'α-synucléine une protéine impliquée dans les formes familiales à transmission autosomique dominante de la maladie a également été démontrée. Ainsi, l'α-synucléine inhibe l'activité de la caspase-3 mais également la voie dépendante de p53 (Alves da Costa et al., 2002). Récemment, une étude portant sur PINK1 (« PTEN-induced kinase-1 ») une protéine impliquée dans les formes récessives de la maladie, a démontré sa capacité à protéger les cellules d'un stimulus apoptotique tel que la staurosporine. En effet, PINK1 a la capacité de réduire l'activité de la caspase-3 et la fragmentation de l'ADN (Petit et al., 2005).

Acronyme	Gène/protéine	Locus	Mode de Transmission	Mutations	Fonction putative	Référence
Formes monogéniques établies						
PARK1/PARK4	*SNCA/ α -synucléine*	4q21-q23	Autosomique dominante	3 mutations faux-sens duplications/triplications	protéine neuronale avec un fonction associée aux vésicules	(Spillantini et al, 1997)
PARK2	*Parkine*	6q25.2-q27	Autosomique récessive	plus de 100 mutations	E3 ubiquitine ligase	(Kitada et al, 1998)
PARK6	*PINK1*	1p35-p36	Autosomique récessive		protéine kinase mitochondriale	(Valente et al, 2004)
PARK7	*DJ-1*	1p36	Autosomique récessive	10 mutations	protéine antioxydante, chaperonne	(Bonifati et al, 2003)
PARK8	*LRRK2/Dardarine*	12q12	Autosomique dominante	16 mutations pathogènes	protéine kinase	(Paisan-Ruiz et al, 2004)
PARK9	*ATP13A2*	1p36	Autosomique récessive	3 mutations (protéines tronquées)	Lysosomale ATPase (10 domaines transmembranaires)	(Ramirez et al, 2006)
Formes monogéniques trouvées dans une seule famille						
PARK5	*UCHL-1*	4p14	Autosomique dominante	1 mutation dans une seule famille	C-terminale ubiquitine hydrolase	(Leroy et al, 1998)
PARK13	*Omi/HtrA2*	2p12	Inconnue	1 mutation dans 4 familles	protéase à sérine	(Strauss et al, 2005)
pas assigné	*Synphiline-1*	5q23.1-q23.3	Inconnue	1 mutation faux-sens	inconnue	(Marx et al, 2003)
pas assigné	*NR4A2/Nurr1*	2q22-q23	Inconnue	3 mutations	membre de la famille des récepteurs nucléaires	(Le et al, 2003)
pas assigné	*POLG*	15q25	Inconnue	1 famille avec des mutations hétérozygotes	ADN polymérase mitochondriale	(Davidzon et al, 2006)
Formes monogéniques relevance inconnue						
PARK3	*Inconnu*	2p23	Autosomique dominante	Inconnue	inconnue	(Gasser et al, 1998)

D'après Klein C, et al, Curr Opin Neurol 2007

Tableau 1: Liste des différentes protéines impliquées dans les formes monogéniques de la maladie de Parkinson

PINK1:PTEN-induced kinase 1, LRRK2: leucine-rich repeat kinase 2, UCH-L1: ubiquitin carboxyterminal hydrolase 1, POLG:

A.VI) Les origines de la maladie de Parkinson :

La majorité des cas de maladie de Parkinson est d'origine sporadique (95%). Les 5% de cas restants sont liés à la présence de mutations dans des régions chromosomiques dont certaines codent pour des protéines identifiées (Tableau 1) (Klein and Lohmann-Hedrich, 2007).

A.VI.1) Les formes sporadiques :

L'origine des formes sporadiques est encore inconnue, cependant l'âge ainsi que des causes environnementales et des facteurs génétiques, dit de susceptibilité, ont été mis en évidence.

A.VI.1.a) Facteurs environnementaux :

De nombreuses études épidémiologiques ont montré un lien entre la vie à la campagne et la maladie de Parkinson. En effet, l'exposition prolongée à différents pesticides et herbicides : tels que les organochloriques (Seidler et al., 1996), le dithiocarbamate (Semchuk et al., 1991), le pyrethroide (Elwan et al., 2006), le manganèse (Huang et al., 1993; Jankovic, 2005), le paraquat (Manning-Bog et al., 2002), la roténone (Betarbet et al., 2000), semble être un facteur de risque (Barbeau 1987). Il a été démontré *in vitro* et dans des modèles animaux que la roténone et le paraquat, en inhibant complexe-I de la mitochondrie, conduisent à l'agrégation de l'α-synucléine (Betarbet et al., 2000; Manning-Bog et al., 2002; Sherer et al., 2002; Sherer et al., 2003).

Chez des toxicomanes la consommation de MPPP un opioïde de synthèse a permis la découverte du MPTP (le 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), en effet, cette molécule peut être produite accidentellement lors de la synthèse du MPPP. Le MPTP induit des symptômes de type Parkinson en seulement trois jours chez l'homme (Langston and Ballard, 1983). Cette molécule est actuellement la plus utilisée pour induire des modèles de maladie de Parkinson *in vitro* et *in vivo*. Ces modèles seront détaillés dans un chapitre leur étant consacré.

A.VI.1.b) Facteurs de susceptibilité :

Les facteurs de susceptibilité sont souvent associés à la variabilité génétique. On peut citer quelques exemples de gènes impliqués dans la survenue de certains cas de maladie de Parkinson à début tardif. Ainsi, une étude a identifié un polymorphisme de la séquence Rep1 du promoteur de l'α-synucléine, induisant une variabilité du taux d'α-synucléine qui prédispose les individus à développer une maladie de Parkinson (Maraganore et al., 2006). C'est également le cas au niveau du promoteur de la parkine (Mata et al., 2004). D'autres travaux ont mis en évidence que des mutations hétérozygotes dans les séquences codant pour la parkine, PINK1 et la dardarine (LRRK2) constituent des facteurs de risque (Di Fonzo et al., 2005; Tan et al., 2007). Ainsi, une haploinsuffisance du gène de l'α-synucléine prédispose au développement d'une maladie de Parkinson (Farrer et al., 2001b).

A.VI.2) Les formes familiales :

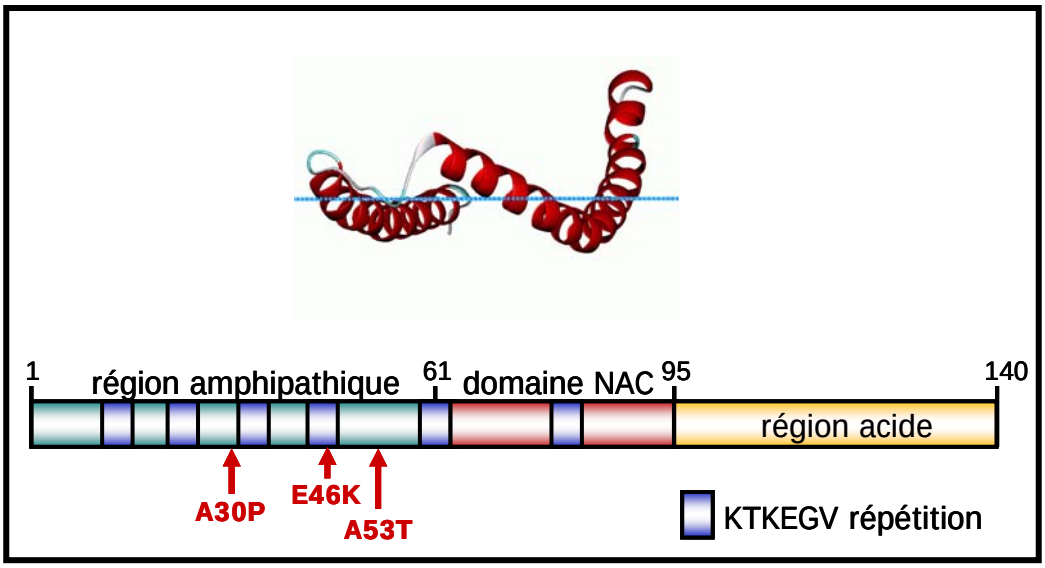
Récemment, des mutations ont été identifiées chez des malades atteints de maladie de Parkinson. Les formes génétiques dues à des mutations situées dans les gènes codant pour l'α-synucléine, l'UCHL1, et LRRK2 se transmettent de façon autosomique dominante. Les mutations situées dans les gènes codant pour la parkine, DJ-1, PINK1 et l'ATP13A2 se transmettent de façon autosomique récessive. Dans quelques familles, des mutations ont également été mises en évidence dans les gènes codant pour la protéine Omi, la synphiline-1, NR4A2 et POLG. Leur mode de transmission reste encore inconnu (Tableau 1).

Durant ma thèse je me suis focalisée sur quelques unes d'entre elles : la synphiline-1, DJ-1 et la parkine. J'ai donc choisi uniquement de vous présenter ces protéines.

A.VI.2.a) L'α-synucléine:

- Généralités :

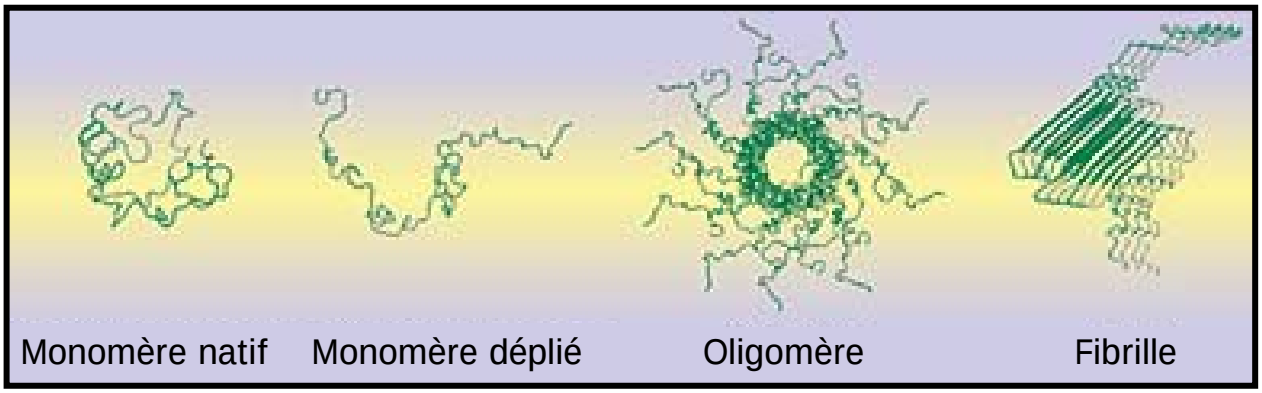
Le gène de l'α-synucléine, SNCA, situé sur le chromosome 4q21-q23, fut le premier identifié comme étant associé aux formes familiales de la maladie de Parkinson (PARK1 et 4) (Polymeropoulos et al., 1997). L'α-synucléine est une protéine de 140 acides aminés, elle est le chef de file des protéines de la famille des synucléines qui compte deux autres membres : la β- et la γ-synucléine. L'homologie de séquences entre ces trois protéines est de 55 à 60% (Clayton and George, 1998; Jakes et al., 1994). L'α-synucléine comprend : une région N-terminale amphipatique contenant 6 séquences répétées imparfaites avec un motif consensus KTKEGV permettant la formation d'une hélice et d'une association stable avec les micelles lipidiques (Davidson et al., 1998; Weinreb et al., 1996), une région centrale



D'après http://opm.phar.umich.edu et Moore D., et al, Annu. Rev. Neurosci. (2005) Vol. 28

Figure 20 : Représentation de la structure 3D et des différents domaines de l'α-synucléine

NAC : "non amyloid component"



D'après Palecek E., et al, Analyst (2008) Vol.133

Figure 21 : Représentation des différentes structures que peut adopter l'α-synucléine

Suivant son environnement l'α-synucléine va changer de structure pour adopter une forme monomérique dépliée, oligomérique ou fibrillaire. L'apparition de certaines de ces formes comme les fibrilles va induire sont agrégation.

hydrophobe contenant le domaine NAC (« non amyloid component ») (Ueda et al., 1993), une région C-terminale acide importante pour son activité de chaperonne (Figure 20) (Kim et al., 2002; Kim et al., 2000) et des motifs caractéristiques des FABP (« fatty acid binding proteines ») (Sharon et al., 2001) importants pour la liaison aux lipides (Perrin 2000). L'α-synucléine est une protéine non structurée mais avec une plasticité conformationnelle importante dûe à la présence du domaine NAC, ce qui permet des changements conformationnels au grè de l'environnement physico-chimique. En effet, elle peut passer d'une conformation non structurée (Weinreb et al., 1996) à une conformation monomérique (El-Agnaf and Irvine, 2002; Perutz et al., 2002; Serpell et al., 2000), oligomérique ou fibrillaire, ce qui peut induire son agrégation (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Ding et al., 2002; El-Agnaf and Irvine, 2000, 2002; Hashimoto et al., 1998; Uversky, 2003) (Figure 21).

- Les mutations :

Depuis la découverte de l'α-synucléine, 3 mutations faux-sens ont été identifiées (Figure 20). La première mutation, SNCA 209G>A (Ala53Thr) (Polymeropoulos et al., 1997), induit un parkinsonisme à début précoce (45,6 ±13,5 ans) et à évolution rapide. La seconde mutation, SNCA 88G>C (Ala30Pro) (Kruger et al., 1998), induit des symptômes parkinsoniens typiques avec un début tardif et une bonne réponse à la dopathérapie (Kruger et al., 2001). Enfin, la troisième mutation SNCA 188G>A (Glu46Lys), est associée à un phénotype de démence à corps de Lewy (Zarranz et al., 2004). En plus de ces mutations faux-sens, on trouve des duplications du gène SNCA conduisant au développement d'un parkinsonisme indistinguable des formes sporadiques (Chartier-Harlin et al., 2004; Nishioka et al., 2006), ainsi que des triplications du gène SNCA (Singleton et al., 2003). La triplication du gène de l'α-synucléine induit un phénotype plus sévère, avec une dégradation rapide, une démence précoce et une réduction de l'espérance de vie. Il existe une variabilité des phénotypes associés aux duplications et triplications (Fuchs et al., 2007; Golbe et al.,

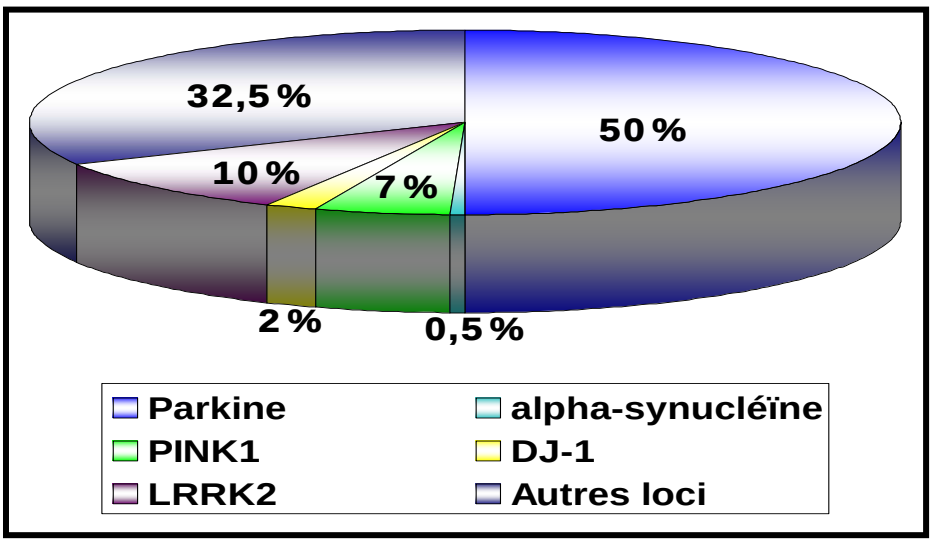
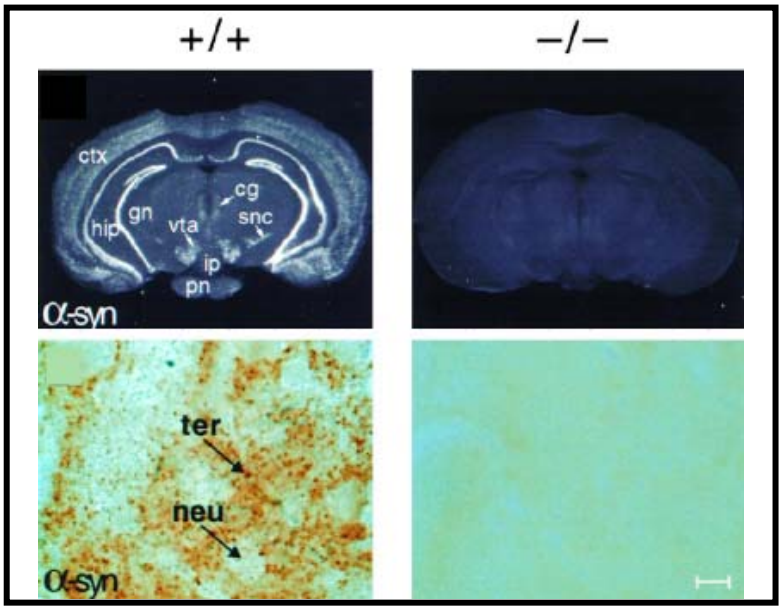


Figure 22 : Estimation de la prévalence de chacune des formes familiales



D'après Abeliovich A., et al, Neuron (2000) Vol. 25

Figure 23 : Expression de l'α-synucléine dans le système nerveux centrale de souris sauvages et de souris invalidées

(gn : corps géniculaire ; cg : noyaux centraux gris ; pn : noyaux pontins ; ip : noyau interpédonculaires ; ter : terminaisons présynaptiques ; neu : neurone)

1996). Ainsi, le phénotype induit par une triplication met en avant l'effet dose, conduisant à un gain de fonction pathologique. Le pourcentage de formes familiales impliquant l'α-synucléine est inférieur à 0,5% des cas (Figure 22), dont une majorité sont des duplications et des triplications.

- Localisation et fonction :

L'α-synucléine est une protéine cytoplasmique dont la fonction physiologique est encore mal connue. Elle est principalement exprimée dans les neurones corticaux, dopaminergiques, noradrénergiques, les cellules endothéliales et les plaquettes, particulièrement dans le néocortex, l'hippocampe, le striatum, le thalamus, et le cervelet (Figure 23) (Abeliovich et al., 2000; Hashimoto et al., 1997; Li et al., 2002; Tamo et al., 2002). Au niveau subcellulaire on trouve l'α-synucléine localisée dans les terminaisons nerveuses présynaptiques, où elle peut être associée de façon réversible aux structures vésiculaires (George et al., 1995; Irizarry et al., 1996; Jensen et al., 2000; Jensen et al., 1999; Jensen et al., 1998; Kahle et al., 2000). Des études d'immunohistochimie ont récemment démontré que la localisation synaptique de l'α-synucléine dépend de son association avec les rafts lipidiques des membranes (Fortin et al., 2004). Toutes ces observations suggèrent un rôle de l'α-synucléine dans la maturation vésiculaire. Les perturbations observées au niveau du recyclage vésiculaire, chez les souris invalidées pour le gène SNAC, associées aux précédentes observations suggèrent fortement une fonction de l'α-synucléine dans ce phénomène. Cette fonction est aussi supportée par des études *in vitro*, qui démontrent que l'α-synucléine peut se lier aux vésicules d'acide phospholipidique (Davidson et al., 1998; Eliezer et al., 2001). Elle peut également se lier et inhiber la phospholipase D (Jenco et al., 1998). De plus, des expériences sur des cultures cellulaires ont montré que l'α-synucléine peut réguler le métabolisme lipidique en protégeant les gouttelettes lipidiques de l'hydrolyse (Cole et al., 2002) et en contrôlant la réserve de vésicules présynaptiques. En effet, la transfection d'un ARN anti-sens dirigé contre l'ARNm de l'α-synucléine réduit cette réserve vésiculaire

Ligand	Références
parkine	Shimura et al, 2001
synphiline-1	Engelender et al, 1999
UCHL-1	Liu et al, 2002
DAT	Torres et al, 2001
TBP-1	Ghee et al, 2000
14-3-3	Ostrerova et al, 1999
Tubuline	Alim et al, 2002
MAP1A/1B	Jensen et al, 2000
Aβ	Yoshimoto et al, 1995
Tau	Jensen et al, 1999
Calmoduline	Lee et al, 2000
ERK	Ostrerova et al, 1999
ERK -1/2	Iwata et al, 2001
complexe ELK-1/ERK-2	Iwata et al, 2001
Protéine kinase C	Ostrerova et al, 1999
kinases SAPK/JNK	Iwata et al, 2001
p38 MAPK	Iwata et al, 2001
Ca^{2+}	Nielsen et al, 2001
Cu^{2+}	Paik et al, 1999, 2000
Zn^{2+}	Kim et al, 2000
Al^{3+}	Paik et al, 1997
Fe^{2+}	Uversky et al, 2001
Cytochrome oxydase	Elkon et al, 2002
Tyrosine Hydroxylase	Perez et al, 2002
Rab5A	Sung et al, 2001
BAD	Ostrerova et al, 1999
PLD2	Jenco et al, 1998

Tableau 2 : Liste non exhaustive des partenaires de l'α-synucléïne

(Murphy et al., 2000). Tout ceci suggère que l'α-synucléine joue un rôle important dans la maturation, le stockage et le transport des vésicules synaptiques. L'invalidation du gène SNAC a confirmé le rôle potentiel de l'α-synucléine dans la régulation de la neurotransmission dopaminergique, puisque les souris sont viables, fertiles et ont une architecture cérébrale normale, mais présentent cependant, une diminution de la libération de dopamine, du taux de dopamine dans le striatum et de la réponse moteur induite par les amphétamines (Abeliovich et al., 2000) (Figure 23).

Le rôle de l'α-synucléine dans la neurotransmission pourrait être également lié à une autre fonction de celle-ci. En effet, une étude a montré une homologie fonctionnelle et structurale (40%) avec la protéine 14-3-3, membre d'une famille de protéines chaperonnes cytoplasmiques ubiquitaires. De plus, l'α-synucléine et la protéine 14-3-3 peuvent interagir avec les mêmes protéines : la protéine kinase C et BAD (Ostrerova et al., 1999). En plus de ces protéines l'α-synucléine est capable de se lier à de nombreux partenaires (Tableau 2), ce qui altère son état de conformation natif et lui permet d'adopter une conformation structurée, caractéristique physique qu'ont les protéines chaperonnes (Uversky et al., 2001a; Uversky et al., 2001b, c, d). Des études *in vitro*, ont également montré une propension de l'α-synucléine à inhiber l'agrégation d'insuline (Kim et al., 2000; Souza et al., 2000). Toutes ces observations suggèrent fortement que l'α-synucléine exerce une fonction de chaperonne.

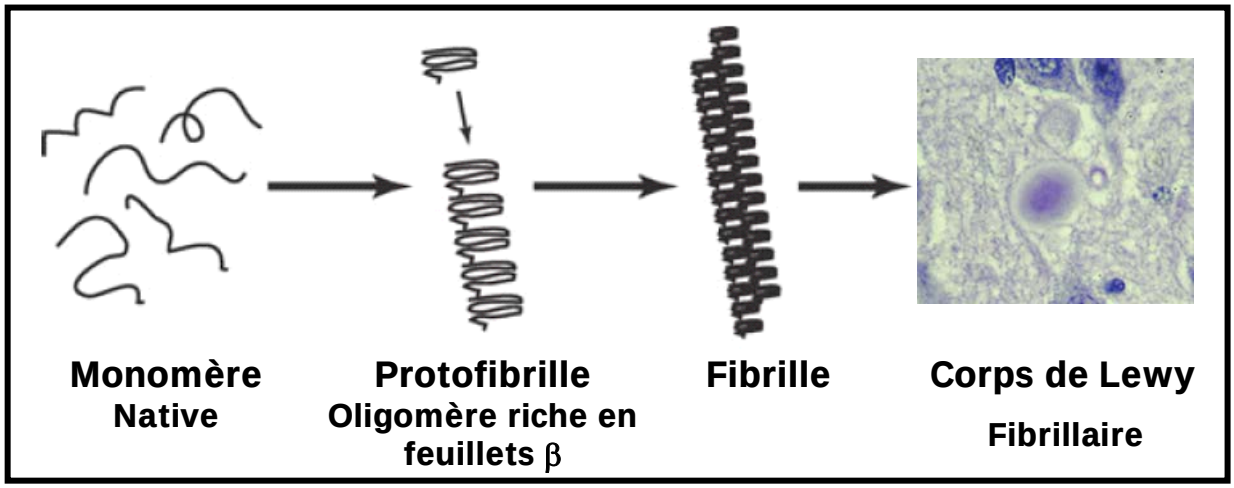
En plus de sa fonction de chaperonne et son implication dans la transmission synaptique, l'α-synucléine joue également un rôle dans les mécanismes d'apoptose. Plusieurs études ont montré que l'α-synucléine pouvait avoir une fonction anti-apoptotique. Ainsi, l'expression de l'α-synucléine sauvage diminue l'activité de la caspase-3 induite par différents stimuli apoptotiques (Alves da Costa et al., 2000; Lee et al., 2001), module l'expression de l'oncogène Bcl-2 (Seo et al., 2002) et inactive la kinase c-Jun impliquée dans la voie de signalisation du stress (Hashimoto et al., 2002). En accord avec ces observations, l'expression de l'α-synucléine est augmentée dans la substance noire à la suite d'une lésion du striatum (zone afférente de la voie dopaminergique nigro-sriatale). A l'inverse d'autres travaux rapportent que l'α-synucléine peut exercer une fonction pro-apoptotique (Iwata et al., 2001b; Sung et al.,

2001). La fonction de l'α-synucléine dans la régulation de la survie cellulaire semble modulée par sa concentration et l'environnement (Xu et al., 2002). A des taux faibles ou élevés, elle peut avoir des fonctions opposées (Seo et al., 2002). Conway et al. ont montré que l'α-synucléine s'agrège à forte concentration (Conway et al., 2000b) ce qui conduit à une perte de fonction et pourrait expliquer les résultats contradictoires rapportés. Ceci fut également confirmé par une étude montrant que l'agrégation et donc le phénotype de l'α-synucléine dépend du stimulus utilisé. En effet, la 6-hydroxydopamine mais pas le MPTP favorise l'agrégation de l'α-synucléine et ainsi sa perte de fonction (Alves da Costa et al., 2006). De plus l'α-synucléine interagit avec différents effecteurs cellulaires impliqués dans le contrôle de la mort cellulaire : la PKC (protéine kinase C), BAD et la kinase Erk (Ostrerova et al., 1999). Ces interactions avec des composants de la machinerie apoptotique comme BAD suggèrent que le phénotype anti-apoptotique de l'α-synucléine pourrait être associé à l'inactivation de ces partenaires et donc à ses capacités de protéine chaperonne.

- La physiopathologie :

L'identification de mutations, de duplications et de triplications du gène SNAC (Chartier-Harlin et al., 2004; Kruger et al., 1998; Polymeropoulos et al., 1997; Singleton et al., 2003), associé au fait que l'α-synucléine sous différentes formes fibrillaire, tronquée, oxydée et/ou phosphorylée (sur la Ser 129) est le composant majeur des corps de Lewy dans la maladie de Parkinson et dans les autres synucléinopathies (Anderson et al., 2006; Spillantini et al., 1998), font de l'α-synucléine une protéine ayant un rôle clé dans la physiopathologie de la maladie de Parkinson.

La triplication du gène SNAC conduit au doublement du niveau d'expression, ce qui démontre que la surexpression de l'α-synucléine est suffisante pour induire la maladie de Parkinson. En effet, des études *in vitro* ont montré que des concentrations supra-physiologiques d'α-synucléine entraînent le passage d'une forme native de la



D'après Moore D., et al, Annu. Rev. Neuroscience (2005) Vol. 28

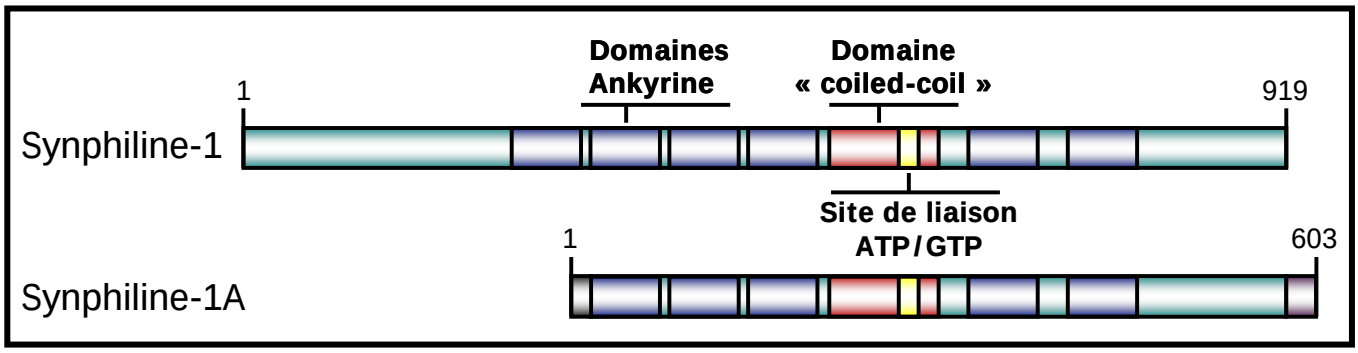
Figure 24 : Fibrillogénèse de l'α-synucléine

Lorsque l'environnement de l'α-synucléine change, sa conformation va évoluer et peut aller jusqu'à l'agrégation. L'α-synucléine peut devenir un composant des corps de Lewy.

protéine à une forme fibrillaire riche en feuillets β (Conway et al., 2000b) qui a une forte propension à l'agrégation. Ceci explique pourquoi la surexpression de l'α-synucléine sauvage dans des modèles cellulaires est toxique. Le fragment C-terminal semble essentiel pour contrôler l'agrégation de l'α-synucléine sauvage. En effet, les souris transgéniques surexprimant une forme tronquée de sa partie C-terminale présentent une perte progressive des neurones dopaminergiques, accompagnée par la présence d'inclusions pathologiques et d'une réduction de la locomotion spontanée apparaissant avec l'âge (Tofaris et al., 2006). Ceci laisse supposer que le domaine C-terminal joue un rôle de régulateur négatif dans l'assemblage des oligomères et suggère que des modifications post-transcriptionnelles telles que l'oxydation, la nitration, ou la phosphorylation, comme la troncation pourraient influencer l'agrégation (Giasson et al., 2000; Hashimoto et al., 1999).

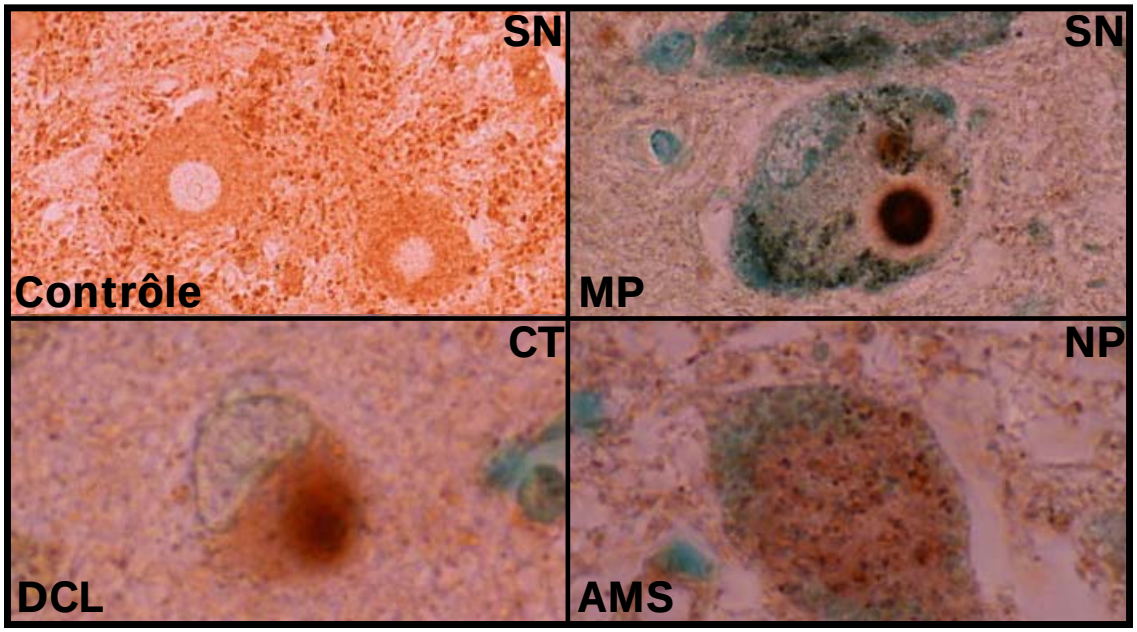
La surexpression de l'α-synucléine dans des cultures cellulaires et particulièrement des formes mutantes est également liée à des déficits mitochondriaux (Hsu et al., 2000), à des perturbations dans la signalisation cellulaire (Gosavi et al., 2002), à une augmentation de l'apoptose (Lee et al., 2001; Zhou et al., 2000), à une perte d'activité de chaperonne (Cuervo et al., 2004), à une élévation de la sensibilité au stress oxydatif (Junn and Mouradian, 2002; Ko et al., 2000) et à une augmentation de la toxicité liée à la dopamine (Tabrizi et al., 2000). Les mécanismes liés à ces différents déficits et l'effet des mutations sur le gène de l'α-synucléine ne sont pas encore clairs. Cependant, on sait qu'elles augmentent les propriétés d'agrégation de l'α-synucléine, conduisant à la formation des espèces oligomeriques (précurseurs des fibrilles) et à des agrégats ressemblants au corps de Lewy *in vitro* (Conway et al., 1998; Giasson et al., 2000; Narhi et al., 1999). Ces mutations favorisent l'oligomérisation mais pas la fibrillation (Conway et al., 2000b) (Figure 24), qui vient ensuite. La toxicité des protofibrilles a été démontrée par une étude sur des souris transgéniques pour l'α-synucléine sauvage. Chez ces souris on observe une perte de motricité et une diminution du nombre de terminaisons nerveuses dopaminergiques, en présence d'inclusions non-fibrillaires d'α-synucléine (Masliah et al., 2000). Cependant lorsque le mutant A30P de l'α-synucléine est surexprimé dans des souris

ou chez la drosophile, la neurodégénérescence n'est observée que lorsque les fibrilles et des inclusions sont présentes (Feany and Bender, 2000; Kahle et al., 2001; Neumann et al., 2002). La contribution des protofibrilles et des fibrilles d'α-synucléine dans la pathogenèse de la maladie de Parkinson reste encore à déterminer.



D'après Szargel R., et al, Cell. Mol. Life Sci. (2008) Vol.65

Figure 25 : Réprésentations schématiques de la structure de la synphiline-1 et de la synphiline-1A



D'après Wakabayashi K., et al, Acta Neuropathologica (2000) Vol.47

Figure 26 : Immunoréactivité de la synphiline-1 dans des cerveaux de patients souffrants de l'une des synucléinopathies

MP : maladie de Parkinson ; DCL : Démence à corps de Lewy ; AMS : atrophie multi-systématisée ; SN : Substance noir ; CT : Cortex temporal ; NP : Noyau pontin

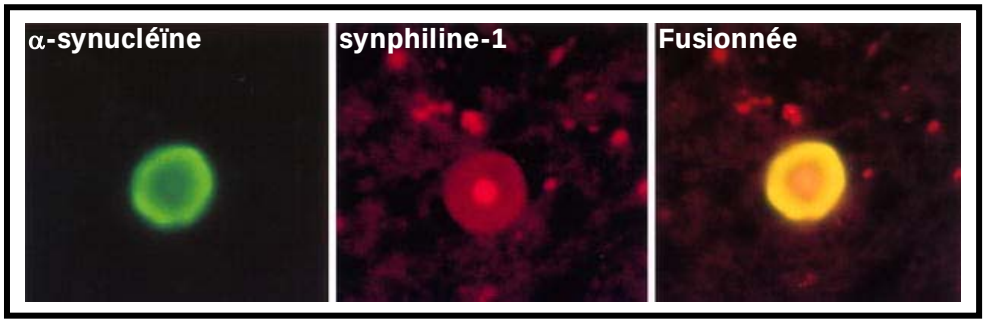
A.VI.2.b) La synphiline-1 :

- Généralités :

Récemment, une expérience de double hybride a permis de mettre en évidence un nouveau partenaire de l'α-synucléine, la synphiline-1, (SNCAIP, « α-synuclein-interacting protein ») (Engelender et al., 1999). Le gène de la synphiline-1 est situé sur le chromosome 5 (5q23.1-23.3) et est composé de 10 exons entrainant la production d'une protéine de 919 acides aminés, avec un poids moléculaire de 115~140 kDa. Dans sa structure on trouve de nombreux domaines d'interaction protéine/protéine, tels que les domaines ankyrine (favorise la reconnaissance moléculaire via des interactions protéine/protéine) et le domaine coiled-coil (domaine composé d'hélices alpha, souvent impliqué dans la multimérisation des protéines) (Figure 25) (Engelender et al., 1999). Dans les cerveaux sains, la synphiline-1 est exprimée de façon ubiquiste, essentiellement dans le périkaryon et les axones des grands neurones (substance noire, cellules pyramidales de l'hippocampe et cellules de Purkinje), au niveau présynaptique où elle est liée aux vésicules synaptiques (Ribeiro et al., 2002). La synphiline-1 interagit également avec les lipides (O'Farrell et al., 2001), présentant un profil de distribution similaire à celui de l'α-synucléine.

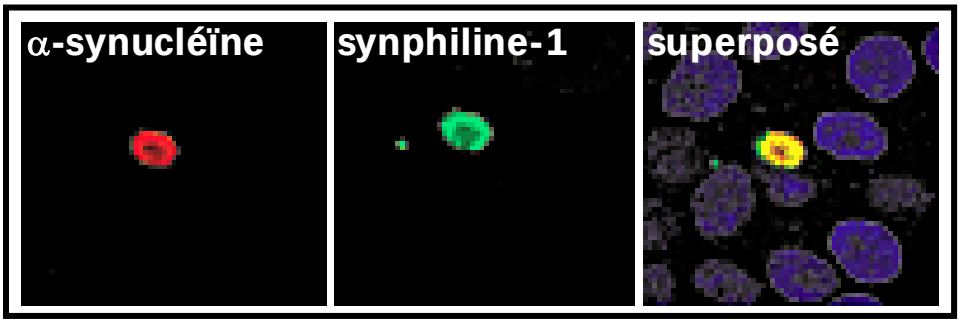
Récemment, l'équipe du Dr Engelender a mis en évidence un nouvel isoforme de la synphiline-1, la synphiline-1A (Eyal et al., 2006), qui est un variant d'épissage, ayant un site d'initiation de lecture différent. La synphiline-1A est composée de : la synphiline-1 moins ses 394 premiers acides aminés (exon 3 et 4), additionnée 28 acides aminés supplémentaires en N-terminal et 51 acides aminés en C-terminal (Figure 25).

Dans les cerveaux de patients parkinsoniens mais également dans les cerveaux de malades souffrant d'autres synucléinopathies, les ARN messagers et la protéine de la synphiline-1 sont localisés dans près de 90% des corps de Lewy, aussi bien au niveau de la substance noire que dans les autres parties du cerveau. Le marquage immunohistochimique est particulièrement intense au niveau du noyau central des



D'après Wakabayashi K., et al, Acta Neuropathologica (2000) Vol.47

Figure 27 : Immunoréactivités de l'α-synucléine et de la synphiline-1 dans des corps de Lewy situés dans la substance noire d'un patient parkinsonien



D'après Chung K., et al, Nature Medicine (2001) Vol. 7

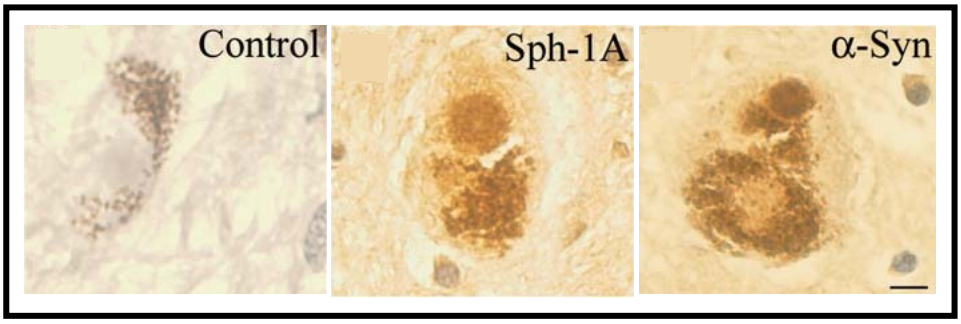
Figure 28 : Immunofluorescences montrant la co-localisation de l'α-synucléine et de la synphiline-1 dans des inclusions cytoplasmiques

Ces photographies montrent la co-localisation de l'α-synucléine et de la synphiline-1 par immunomarquage dans des inclusions cytoplasmiques. La co-expression de ces deux protéines induits la formation de ces inclusions dans des cellules.

corps de Lewy (Figure 26) (Engelender et al., 2000; Humbert et al., 2007; Murray et al., 2003; Ribeiro et al., 2002; Wakabayashi et al., 2002). De façon intéressante, un co-marquage synphiline-1, α-synucléine montre une co-localisation parfaite de ces deux protéines dans les corps de Lewy (Figure 27). Une mutation sur la synphiline-1 a été récemment identifiée (R621C) chez deux patients souffrant de la maladie de Parkinson (Marx et al., 2003). Ces observations soulignent l'importance de cette protéine dans le développement de la maladie. La fonction de la synphiline-1 reste encore à déterminer.

- Formation des corps de Lewy :

Interaction avec différentes protéines associées à la maladie de Parkinson : Plusieurs études ont confirmé l'interaction physique et la co-localisation de la synphiline-1 et de l'α-synucléine *in vitro* et *in vivo* (Engelender et al., 1999; Engelender et al., 2000; Kawamata et al., 2001; Murray et al., 2003; Ribeiro et al., 2002; Wakabayashi et al., 2002). Différentes régions de la synphiline-1 sont impliquées dans l'interaction avec l'α-synucléine. Ainsi l'équipe du Dr Kawamata a démontré que les domaines C-terminaux des deux protéines interagissent étroitement (Kawamata et al., 2001). Cependant, une autre équipe, celle du Dr Neystat a montré par une expérience de double hybride, que le domaine central de la synphiline-1 (acide aminé 349 à 555) est nécessaire et suffisant à l'interaction avec le domaine 1 à 65 de l'α-synucléine (Neystat et al., 2002). Une observation majeure vient supporter l'idée d'une fonction importante de la synphiline-1 dans la maladie de Parkinson. En effet, la co-expression de ces deux protéines dans des cellules en culture conduit à la formation d'inclusions cytoplasmiques semblable aux corps de Lewy (Chung et al., 2001; Engelender et al., 1999; Lee et al., 2004) (Figure 28). Ce résultat suggère un rôle de la synphiline-1 dans la formation des corps de Lewy. Des travaux récents ont de plus montré sa co-localisation avec l'α-synucléine accumulée dans les cerveaux de souris transgéniques homozygotes pour la mutation A53T de l'α-synucléine (Shirakashi et al., 2006).



D'après Eyal A., et al, PNAS (2006) Vol. 103

Figure 29 : Immunomarquage de la synphiline-1A et de l'α-synucléine dans les corps de Lewy de la substance noire d'un patient parkinsonien.

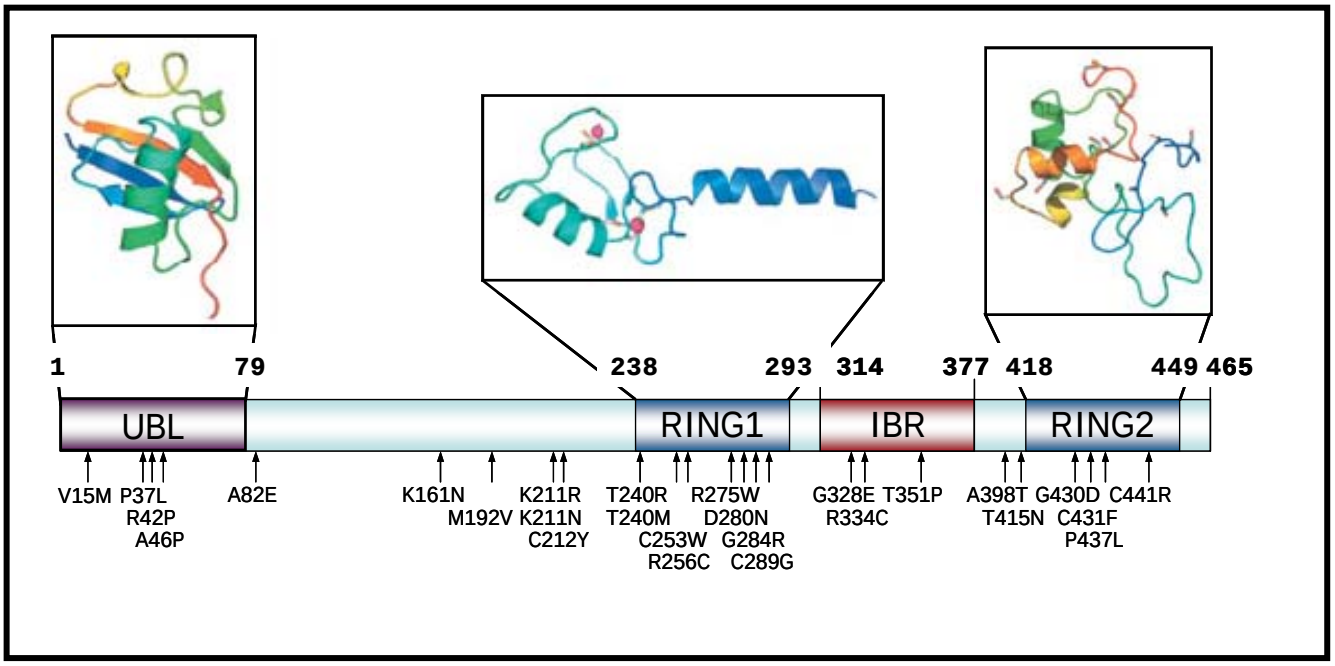
Tout comme la synphiline-1 l'isoforme 1A est présente dans les corps de Lewy des différentes synucléinopathies, et est capable d'interagir avec l'α-synucléine (Eyal and Engelender, 2006) (Figure 29). De plus cet isoforme présente des capacités d'agrégation différentes de celles de la synphiline-1. La transfection de la synphiline-1A dans des cellules SH-SY5Y, induit la formation d'agrégats dans le cytosol, alors que la synphiline-1 n'induit pas cette agrégation. Ceci laisse supposer que le domaine ankyrine manquant contrôle l'agrégation de la protéine (Eyal and Engelender, 2006). L'expression de la synphiline-1A induit une neurotoxicité et de façon très intéressante cette toxicité est atténuée lorsqu'il y a formation d'inclusions (Eyal et al., 2006). Ceci suggère fortement un rôle cytoprotecteur de ces inclusions.

Ubiquitinylation/phosphorylation : La synphiline-1 est ubiquitinylée et dégradée par le système ubiquitine-protéasome (Lee et al., 2002b; Liani et al., 2004). En effet, la synphiline-1 interagit et est ubiquitinylée par différentes ubiquitine E3 ligases : la parkine, la dorfine, SIAH-1 et SIAH-2 (Chung et al., 2001; Ito et al., 2003; Liani et al., 2004; Nagano et al., 2003). De façon intéressante, l'ubiquitinylation par SIAH-1 et SIAH-2 conduit à la dégradation de la synphiline-1 par le protéasome, mais ce n'est pas le cas lorsqu'elle est ubiquitinylée par la parkine (Ito et al., 2003; Lim et al., 2005), ce qui promeut la formation d'inclusions. La parkine est une des protéines identifiées comme étant impliquée dans les formes familiales de la maladie (le prochain chapitre de cet exposé lui est consacré). La synphiline-1 interagit aussi avec une autre protéine impliquée dans le système ubiquitine-protéasome, NUB1 (« NEDD8 Ultimate Buster-1 ») une protéine régulatrice connue pour augmenter la dégradation d'une autre protéine NEDD8 (« Neural precursor cell expressed, developmentally down-regulated 8 »). Il semble que NUB1 favorise l'adressage de la synphiline-1 au protéasome (Kamitani et al., 2001; Tanji et al., 2006). Liani et collaborateurs ont montré que l'ubiquitinylation de la synphiline-1 est nécessaire à la formation d'inclusions cytoplasmiques. Si le protéasome est inhibé alors que la synphiline-1 est ubiquitinylée par SIAH-1, on observe la formation d'inclusions cytoplasmiques (Liani et al., 2004).

La régulation de la synphiline-1 passe également par des phosphorylations. En effet, la synphiline-1 interagit avec 3 différentes kinases. La première est la caséine kinase 2 qui phosphoryle la synphiline-1 *in vivo*, induisant une diminution de l'interaction synphiline-1/α-synucléine et donc de la formation d'inclusions (Lee et al., 2004). La seconde est la GSK3β qui phosphoryle *in vitro* et *in vivo* la synphiline-1 sur la sérine 556, cette phosphorylation induit la diminution de l'ubiquitinylation de la synphiline-1 et la formation d'inclusions (Avraham et al., 2005). La troisième kinase est une autre protéine impliquée dans les formes familiales de la maladie LRKK2 (ou dardarine) qui interagit également avec la parkine et l'α-synucléine, ces protéines pourraient former un complexe multi-protéique (Smith et al., 2006). La synphiline-1 est également régulée à un autre niveau. Ainsi, l'α-synucléine peut être phosphorylée sur sa sérine 129 favorisant l'interaction α-synucléine/synphiline-1, ce qui induit une diminution de l'ubiquitinylation de la synphiline-1 et l'augmentation de la formation d'inclusions (Smith et al., 2005a). On peut noter aussi que la phosphorylation de la parkine sur sa sérine 135 par Cdk5 entraine une diminution de son auto-ubiquitinylation, et de ce fait une diminution de sa capacité à ubiquitinyler la synphiline-1, provoquant une diminution de la formation d'inclusions (Avraham et al., 2007). Toutes ces observations démontrent l'importance de l'implication de ces kinases directement ou indirectement sur la régulation de l'ubiquitinylation de la synphiline-1, contribuant ainsi à la formation des corps de Lewy.

- Régulation de l'activité du protéasome

La relation entre la synphiline-1 et le protéasome ne s'arrête pas là, il semble que la surexpression de la synphiline-1 induit l'inhibition de l'activité du protéasome (Avraham et al., 2005; Kalia et al., 2004). Une étude du Dr Marx montre également une interaction entre la synphiline-1 et la protéine ATPase S6, la surexpression de ces deux protéines entraîne une diminution de l'activité du protéasome et une augmentation du nombre d'inclusions (Marx et al., 2007). Ceci ajouté à la précédente observation suggère une nouvelle fonction de la synphiline-1 : la régulation directe de la fonction du protéasome.



D'après Bossy-Wetzel E., et al, Nature Medecin. (2004) Vol. 10

Figure 30 : Représentation de la structure et des différents domaines de la parkine

"UBL : Ubiquitin-like domain; IBR : in-between RING finger domain; RING : really interesting new gene."

A.VI.2.c) La parkine :

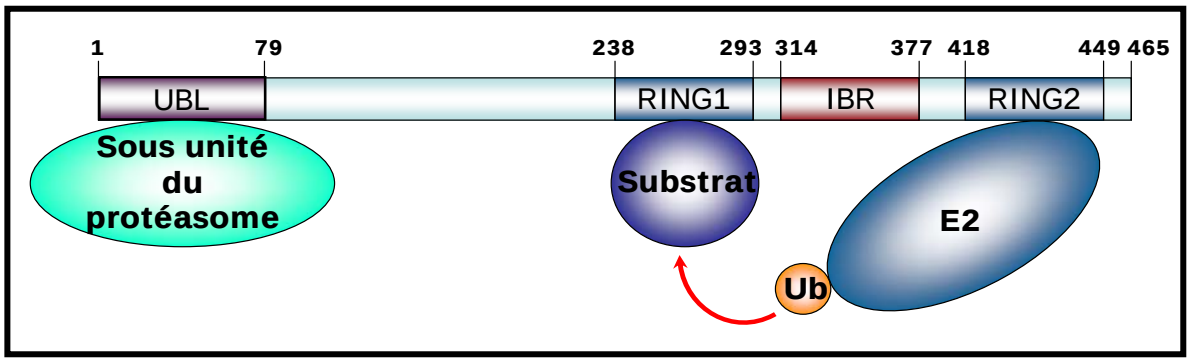
- Généralités :

C'est en 1998 que l'équipe du Dr. Kitada a mis en évidence l'implication de la parkine (PARK2) dans les formes à transmission autosomique récessive de la maladie, dans une famille Japonaise présentant une forme juvénile de maladie Parkinson (Kitada et al., 1998). Le gène de la parkine est localisé sur le chromosome 6q25.2-27, il comprend 12 exons associés à une région intronique très étendue d'1,3MB, et code pour une protéine de 465 acides aminés. La structure de la parkine comprend une région centrale, qui lie un domaine N-terminal UBL (« ubiquitine-like domain ») possédant 62% d'homologie avec la séquence de l'ubiquitine, une région C-terminale comprenant un domaine RBR : 2 domaines à doigt de zinc de type RING (« Really Interesting New Gene ») et un domaine IBR (« in-between-RING »). Ces différents domaines permettent des interactions protéine/protéine (Figure 30) (Marin et al., 2004; Morett and Bork, 1999; Shimura et al., 2000). La parkine est principalement cytoplasmique, mais on la retrouve également en faible quantité au niveau de la membrane mitochondriale externe (Darios et al., 2003).

- Fonction :

La parkine est une ubiquitine ligase E3 (Shimura et al., 2000; Zhang et al., 2000), qui participe au système ubiquitine-protéasome (Figure 13), l'ubiquitine ligase E3 détermine le substrat à ubiquitinyler, c'est elle qui confère sa spécificité au système. En effet, elle doit à la fois interagir avec enzyme de conjugaison E2 et la protéine substrat pour catalyser le transfert de l'ubiquitine sur le substrat.

La parkine interagit par ses domaines RING avec des enzymes de conjugaison E2 spécifiques : UbcH7 et UbcH8 (Shimura et al., 2000; Zhang et al., 2000), et les protéines UBC6 et UBC7 qui sont des enzymes E2 associées au réticulum endoplasmique (Imai et al., 2001). Le domaine UBL de la parkine interagit avec la



D'après Bossy-Wetzel E., et al, Nature Medecin (2004) Vol. 10

Figure 31 : Représentation schématique des interactions entre la parkine et ces partenaires

Ub : ubiquitine

Substrats putatifs	Contrôle du taux	Ubiquitination par la parkine	Altéré dans les cerveaux AR-JP
CDCrel-1	oui	oui (polyubiquitination)	oui, ↑
CDCrel-2a	inconnu	oui	oui, ↑
O-glycosylated α-synucléïne	inconnu	oui (polyubiquitination)	oui, ↑
Cycline E	oui	oui (polyubiquitination)	oui, ↑
Synphiline-1	non	oui (polyubiquitination)	non
Pael-R	oui (protéasome)	oui (polyubiquitination)	oui, ↑
p38/ JTV-1	oui (protéasome)	oui (mono ou polyubiquitination)	oui, ↑
FBP1	oui	oui	oui, ↑
α/ β-tubuline	oui	oui	inconnu
RanBP2	oui (protéasome)	oui (polyubiquitination)	inconnu
Hsp70	non	oui (monoubiquitination)	non
Synaptotagmin XI	oui	oui (polyubiquitination)	inconnu

D'après Moore D., Biochem. Soc. Trans (2006) Vol. 34

Tableau 3 : Liste des différents substrats de la parkine

« CDCre-1/2 : cell division control-related protein 1/2 ; Pael-R : parkin-associated endothelin-like receptor ; FBP1 : far upstream sequence element-binding protein 1 ; RanBP2 : Ras-related small nuclear protein binding protein 2; Hsp70: Heat Shock Protein. »

sous-unité Rpn10 (19S) du protéasome 26S via son arginine en position 42 (Sakata et al., 2003). Rpn1 est une sous-unité qui intervient dans le transfert des substrats poly-ubiquitinylés vers le protéasome (Figure 31). L'activité ubiquitine ligase E3 de la parkine peut-être amplifiée lorsque la parkine interagit avec les complexe SCF-like (« Skp1-Cullin-F-box protein ») ou Hsp70/CHIP favorisant la dégradation de certains de ces substrats (cycline E), ce qui tend à suggérer que la parkine agit à l'intérieur d'un complexe multi protéique (Imai et al., 2002) (Staropoli et al., 2003).

Des expériences *in vitro* et sur des cellules en culture ont permis de mettre en évidence différents substrats de la parkine qu'elle peut ubiquitinyler afin de réguler ou pas leur taux. Parmi ces protéines on trouve CDCrel-1 (« cell division control related protein 1 »), une protéine associée aux vésicules synaptiques et impliquée dans la régulation de la neurotransmission (Zhang et al., 2000), ainsi que de rares formes d'α-synucléine O-glycosylées (Shimura et al., 2001). Les protéines Pael-R (« Parkin-associated endothelin-like Receptor ») (Imai et al., 2001), α/β-tubuline (Ren et al., 2003), FBP1 (« Far upstream sequence element-Binding Protein 1 ») (Ko et al., 2006), RanBP2 (« Ras-related small nuclear protein Binding Protein 2 ») (Um et al., 2006), et Hsp70 une protéine chaperonne (« Heat shock protein »), sont également des substrats de la parkine. Tout comme la synaptotagmine XI, une protéine impliquée dans la maintenance de la fonction synaptique (Huynh et al., 2003), la cycline E impliquée dans le cycle cellulaire (Staropoli et al., 2003) et p38/JTV-1 une sous-unité du complexe aminoacyl-tARN synthétase (Corti et al., 2003) (Tableau 3). Il faut remarquer que le nombre de substrats protéiques de la parkine semble élevé. La pertinence physiologique de ces interactions reste à démontrer dans des études *in vivo*, en effet plusieurs d'entre eux ne s'accumulent ni dans les cerveaux de souris déficientes en parkine ni dans les cerveaux de patients.

La parkine parait également être un modulateur clé de la fonction mitochondriale. En effet la parkine semble jouer un rôle dans la morphogenèse mitochondriale durant la spermatogenèse (Riparbelli and Callaini, 2007), et induire une augmentation de la biogenèse mitochondriale dans les cellules qui prolifèrent (Kuroda et al., 2006). La parkine est aussi capable de restaurer les dysfonctions

mitochondriales, la dégénérescence musculaire et la perte neuronale observée dans des drosophiles suite à l'invalidation de PINK1 (sérine/thréonine kinase mitochondriale) (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). De plus, l'invalidation du gène de la parkine chez la drosophile entraine une réduction de l'espérance de vie, des défauts moteurs, une stérilité des mâles (Greene et al., 2005; Pesah et al., 2004). De même chez des souris invalidées pour le gène de la parkine on observe une augmentation du stress oxydatif dû à un défaut mitochondrial, et une perte neuronale (Goldberg et al., 2003; Palacino et al., 2004). Ces différentes études confirment donc la fonction mitochondriale de la parkine *in vivo*.

Ces travaux suggèrent également une fonction protectrice de la parkine. Plusieurs données montrent que la parkine est capable de protéger les cellules contre différents agents stressants et toxiques (Feany and Pallanck, 2003), les mécanismes impliqués dans cette fonction sont mal connus. En effet, dans différents types cellulaires, la surexpression de la parkine confère aux cellules une résistance contre :

- l'apoptose impliquant la voie mitochondriale (Darios et al., 2003), ou due à l'inhibition du protéasome (Petrucelli et al., 2002),

- la toxicité induite par la dopamine en diminuant le stress oxydatif, ce qui pourrait relier la parkine à la survie des neurones dopaminergiques (Jiang et al., 2004),

- l'excitotoxicité lors du traitement de neurones primaires au kaïnate, en supprimant vraisemblablement l'accumulation de cycline E (Staropoli et al., 2003),

- la mort cellulaire qui suit la surexpression de nombreuses protéines dont des substrats de la parkine : Pael-R (Imai et al., 2001), p38 (Corti et al., 2003), CDCrel-1 (Son et al., 2005), mutants de l'α-synucléine (Petrucelli et al., 2002), et mutants de la protéine Tau (Klein et al., 2006; Menendez et al., 2006),

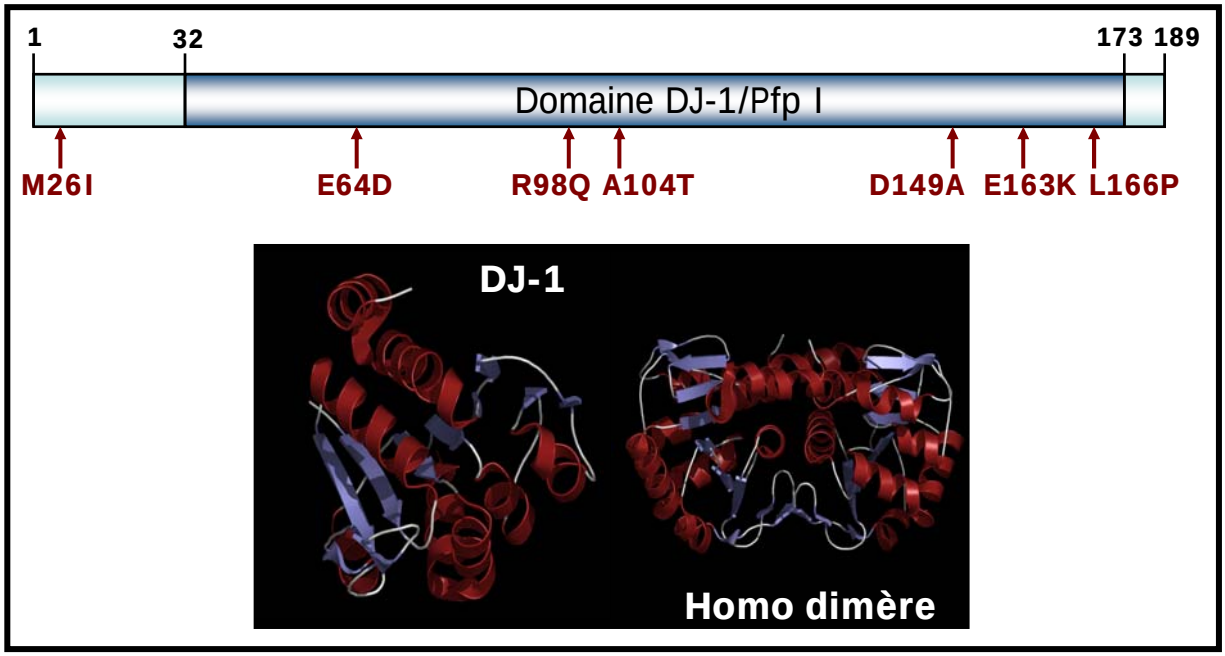
- l'apoptose provoquée par des toxines mitochondriales (MPP^+, roténone) (Casarejos et al., 2006; Hyun et al., 2005), ou du manganèse (Higashi et al., 2004).

Ces différents travaux suggèrent que les capacités assez larges de neuroprotection de la parkine sont liées à sa capacité à mono ou à poly-ubiquitinyler ses substrats, et indiquent que la parkine exerce un rôle de ménage et de maintenance afin de conserver l'ubiquitinylation et les fonctions du protéasome dans les neurones.

- Les mutations et la physiopathologie :

Les formes génétiques précoces (avant 50 ans) de la maladie de Parkinson sont majoritairement (50%) induites par des mutations sur le gène de la parkine (Figure 31). La parkine est également responsable de près de 20% des formes sporadiques précoces de la maladie de Parkinson. Actuellement, plus de 100 mutations différentes sont connues sur le gène de la parkine, parmi elles des délétions pouvant aller jusqu'à plusieurs Kilo-bases, des multiplications génomiques et des mutations faux sens (Figure 30) (Lucking et al., 2000; Mata et al., 2004; West and Maidment, 2004). Ces mutations sur le gène de la parkine induisent les signes cliniques classiques d'une maladie de Parkinson, mais avec un début précoce et une évolution lente de la maladie, une dystonie précoce et l'apparition d'hyper réflexes (Lohmann et al., 2003).

Les patients parkinsoniens présentant une mutation au niveau du gène de la parkine ne développent pas de corps de Lewy (Farrer et al., 2001a). Cependant, dans les cas de formes sporadiques ou génétiques induites par l'α-synucléine, la parkine peut être localisée dans les corps de Lewy (Pramstaller et al., 2005; Schlossmacher et al., 2002). Les différentes mutations sur la parkine induisent différents phénotypes dont une perte de son activité ligase, ce qui affecte l'ubiquitinylation et la dégradation de ses substrats pouvant provoquer leur agrégation (Imai et al., 2000; Zhang et al., 2000), mais également des perturbations au niveau de sa solubilité, sa localisation et des interactions avec ses partenaires (Hampe et al., 2006; Matsuda et al., 2006; Sriram et al., 2005).



D'après Moore D., et al, Annu. Rev.Neurosci. (2005) Vol 28, et

www.lifesci.sussex.ac.uk/research/bioinformatics

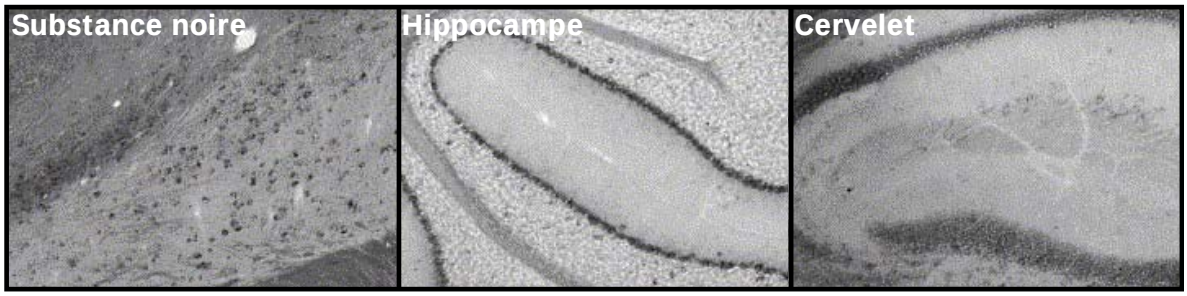
Figure 32 : Représentations schématiques de la structure linéaire et en 3 dimensions de DJ-1

A.VI.2.d) DJ-1

- Généralités :

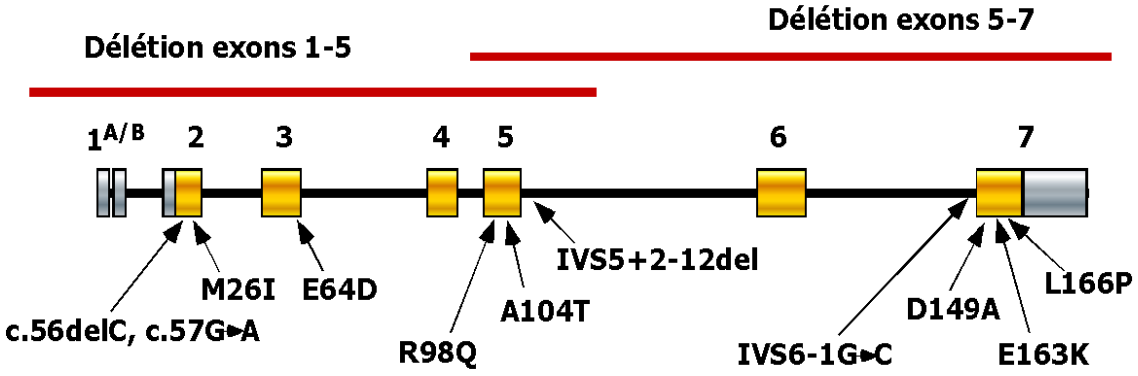
En 2003, le Dr. Bonifati et collaborateurs ont associé le locus PARK7 situé sur le chromosome 1p36 (van Duijn et al., 2001) à la protéine, DJ-1 (Bonifati et al., 2003). Le gène de DJ-1, comprend 8 exons dont les exons 1a/b qui ne sont pas transcrits suggérant un épissage alternatif (Abou-Sleiman et al., 2004; Taira et al., 2001). Les exons 2 à 7 codent pour une protéine de 189 acides aminés très conservée dans de nombreuses espèces (Bandopadhyay and Cookson, 2004; Nagakubo et al., 1997) (Figure 32). Les séquences des protéines humaine et murine ont 90% d'homologie. Il existe également une homologie entre les séquences de DJ-1, celles des protéines HSP chaperonnes et celles des protéases à cystéine Thij/PfpI. Pourtant, DJ-1 ne semble pas posséder d'activité de protéase à cystéine (Halio et al., 1996; Mizote et al., 1996). La cristallisation de DJ-1 a permis de mettre en évidence une structure homodimérique (Huai et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003), structure confirmée dans des cultures cellulaires (Miller et al., 2003; Moore et al., 2003b) (Figure 32).

DJ-1 est une protéine ubiquitaire fortement exprimée aussi bien dans le cerveau que dans les organes périphériques. Elle est exprimée dans tous les tissus que ce soit chez la souris ou chez l'homme, avec une prédominance dans les testicules, le rein et le cerveau (Nagakubo et al., 1997; Olzmann et al., 2004). Différentes études sur des cerveaux murins ont mis en évidence une localisation des ARNm et la protéine à la fois dans des cellules neuronales et des cellules gliales (astrocytes, microglie, oligodendrocytes). La forte expression de DJ-1 dans ces différents types cellulaires n'est pas confinée à un seul système (GABAergique, glutamatergique, dopaminergique, cholinergique) ni à une seule région anatomique. En effet, l'immunoréactivité de DJ-1 est retrouvée dans des structures liées au système moteur telles que la substance noire, le putamen, le noyau caudé, le pallidium, le noyau rouge et les noyaux profonds du cervelet. Elle est également présente dans des structures liées aux systèmes non moteurs telles que l'hippocampe,



D'après Bader V., Brain Res. (2005) Vol.1041

Figure 33 : Distribution des ARNm de DJ-1 dans le cerveau de souris adultes



Mutations	Origines	Gène	Protéine	Références
Leu166Pro	Italie	Mutation ponctuelle homozygote	Perte de la stabilité de la protéine	Bonifati et al, 2003
Délétion exons 1-5	Hollande	Délétion de 14kb homozygote	Pert de la protéine	Bonifati et al, 2003
Met26Ile	Juifs ashkénase	Mutation ponctuelle homozygote	Inconnue	Abou-Sleiman et al, 2003
Asp149Ala	Carraibe	Mutation ponctuelle hétérozygote	Inconnue	Abou-Sleiman et al, 2003
Ala104Thr	Latino	Mutation ponctuelle hétérozygote	Inconnue	Hague et al, 2003
IVS 6-1 G→C	Espagnole	Mélange hétérozygote	Protéine abérante	Hague et al, 2003
c.56delC c.57G→A	Espagnole	Mélange hétérozygote	Protéine tronquée	Hague et al, 2003
IVS 5+2-12del	Russe	Hétérozygote	Protéine abérante	Hedrich et al, 2004
Délétion exons 5-7	Italie du nord	Délétion hétérozygote	Protéine abérante	Hedrich et al, 2004
Arg98Gln	Multiple	Hétérozygote	Inconnue	Hague et al, 2003
Glu64Asp	Turque	Homozygote	Inconnue	Bonifati 2004
Glu163Lys	Italie	Homozygote	Inconnue	Amnesi et al, 2005
g.168_185dup	Italie	Homozygote	Inconnue	Amnesi et al, 2005

Figure 34 : Schéma et tableau récapitulant des mutations que l'on peut trouver sur le gène de DJ-1

les bulbes olfactifs, le noyau réticulaire du thalamus et le cortex piriforme (Bader et al., 2005; Bandopadhyay et al., 2005; Kotaria et al., 2005; Shang et al., 2004) (Figure 33). La prédominance de DJ-1 dans les différentes régions du cerveau est corrélée non seulement à sa présence dans les régions cérébrales liées aux symptômes moteurs mais également aux symptômes non moteurs de la maladie. Des études sur la distribution subcellulaire de DJ-1 ont montré que cette protéine est présente dans le noyau, dans le cytoplasme et dans la mitochondrie (Nagakubo et al., 1997; Zhang et al., 2005). Il est intéressant de noter qu'en condition de stress, le taux de DJ-1 mitochondriale augmente (Blackinton et al., 2005; Canet-Aviles et al., 2004). Tout ceci pourrait rendre compte des symptômes observés lorsque DJ-1 est mutée.

- Les mutations et la physiopathologie:

Les premières mutations identifiées sur le gène de DJ-1 furent une délétion homozygote des exons 1 à 5 dans une famille allemande, et une mutation faux-sens homozygote Leu166Pro (L166P) dans une famille Italienne (Bonifati et al., 2003) Plusieurs autres mutations furent identifiées par la suite (Abou-Sleiman et al., 2003; Annesi et al., 2005; Bonifati et al., 2004; Hague et al., 2003; Hedrich et al., 2004) (Figure 34). Les formes de la maladie de Parkinson liées aux mutations sur le gène de DJ-1 sont à transmission autosomique récessive et représentent environ 1 à 2% des formes précoces (Figure 21) (Abou-Sleiman et al., 2003; Clark et al., 2004; Hedrich et al., 2004; Klein et al., 2005; Lockhart et al., 2004). A ce jour aucune mutation sur DJ-1 n'a été identifiée dans les formes sporadiques (Abou-Sleiman et al., 2003). Par contre dans ces mêmes formes sporadiques on observe une très forte augmentation du taux de formes insolubles de DJ-1 (Moore et al., 2005b). De façon intéressante, DJ-1 n'est pas ou est faiblement exprimée dans les corps de Lewy (Bandopadhyay et al., 2004; Rizzu et al., 2004).

Sur un plan clinique, les formes de la maladie de Parkinson liées à DJ-1 sont caractérisées par un début précoce (pouvant être inférieur à 30 ans), une progression lente de la maladie, et une bonne réponse à la levodopa. On peut observer des

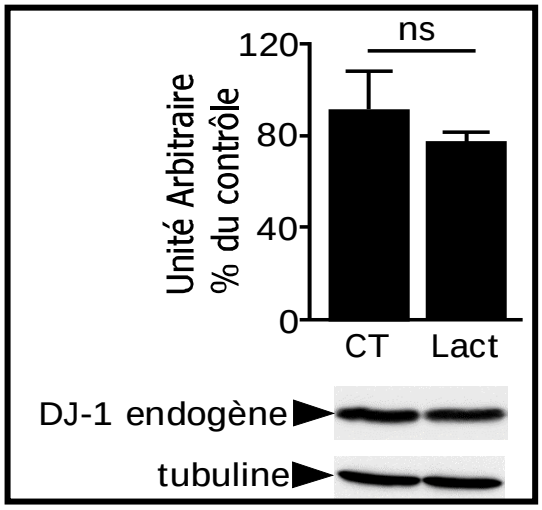


Figure 35 : Expression de DJ-1 endogène dans des cellules neuronales après traitement à la lactacystine

Mesure de l'expression de DJ-1 endogène dans des cellules neuronales murines en conditions contrôle ou traité à la lactacystine (un inhibiteur du protéasome) par immunoempreinte. Le graphique illustre l'analyse densitométrique de 8 expériences indépendantes ; ns : non significatif ; lact : lactacystine.

troubles psychiatriques (anxiété et épisodes psychotiques), des troubles du comportement et une dystonie de fonction (incluant des blépharospasmes) (Abou-Sleiman et al., 2004).

La stabilité de DJ-1 dépend de sa capacité à former un dimère. La mutation L166P affecte cette capacité, ce qui induit une augmentation de la dégradation de DJ-1 par le protéasome abolissant ainsi la fonction antioxydante de DJ-1 (Miller et al., 2003; Moore et al., 2003b; Olzmann et al., 2004). La dégradation de DJ-1 par le protéasome est sujette à controverse, en effet, Görner et collaborateurs ont montré que des inhibiteurs du protéasome n'ont aucun effet sur le métabolisme de DJ-1 (Görner et al., 2004), ce que nous avons également pu observer (Figure 35) lorsque l'on regarde l'expression de la protéine endogène. Cette controverse au sujet de la stabilité de DJ-1 peut s'expliquer. La protection de DJ-1 par des inhibiteurs du protéasome n'est visible que lorsque l'on est en conditions de surexpression, en effet ces inhibiteurs ont tendance à augmenter l'activité du promoteur CMV qui contrôle la transcription des protéines dans les constructions qui sont surexprimées (Dunys et al., 2006). La mutation L166P entraîne également des changements au niveau subcellulaire, en augmentant la localisation mitochondriale de DJ-1 au détriment de sa localisation cytoplasmique (Zhang et al., 2005). DJ-1 subit aussi des modifications post-traductionnelles, telle qu'une sumoylation par les protéines PIASxα ou PIASy sur sa lysine en position 130. Il existe un processus de balance entre la sumoylation et l'ubiquitinylation permettant de réguler l'équilibre entre dégradation et translocation des protéines au noyau (Hegde and DiAntonio, 2002). La sumoylation de DJ-1 suggère donc un rôle de cette protéine dans la signalisation cellulaire. De plus, la mutation L166P qui induit un défaut de sumoylation de DJ-1 entraine une augmentation de son insolubilité, une diminution de son adressage à la mitochondrie et une augmentation de sa dégradation par le protéasome (Shinbo et al., 2006).

Dans les cerveaux de patients atteints par les formes sporadiques de la maladie, DJ-1 est oxydée et endommagée de façon irrémédiable par une carbonylation. DJ-1 est également susceptible d'être oxydée sur ses méthionines (Choi et al., 2006).

- Les fonctions de DJ-1 :

Oncogène : Tout d'abord, DJ-1 fut identifiée comme étant un oncogène favorisant la transformation des cellules NIH3T3 en coopérant avec Ras (Nagakubo et al., 1997). De plus, DJ-1 transréprime PTEN un des suppresseurs de tumeur les plus souvent muté dans les cancers humains (Cantley and Neel, 1999; Kim et al., 2005a). Il a également été rapporté une augmentation du niveau de DJ-1 dans plusieurs cancers notamment des poumons, de la prostate et du sein. Dans ce dernier cas DJ-1 a d'ailleurs été montrée comme étant un bon biomarqueur, en effet, chez les patients un fort taux de DJ-1 a été mesuré dans la circulation sanguine quand on les compare à des sujets contrôles (Hod, 2004; Kim et al., 2005a; Le Naour et al., 2001; Sekito et al., 2005). Cependant, le rôle de DJ-1 dans la transformation des cellules n'est pas sa seule fonction.

DJ-1 et fertilité : Le fait que DJ-1 soit localisée au niveau des testicules, associé au fait que sa protéine homologue chez le rat est CAP1 (« contraception associated protein »), suggère que DJ-1 pourrait également être impliquée dans la fertilité masculine chez l'homme (Yoshida et al., 2003). Des études ont montré une corrélation entre les niveaux de CAP1/DJ-1 dans le sperme et l'épididyme, et l'infertilité masculine après des traitements toxiques (Klinefelter and Suarez, 1997; Wagenfeld et al., 1998; Welch et al., 1998). De plus, DJ-1 peut activer le récepteur aux androgènes, un membre de la superfamille des récepteurs nucléaires impliqués dans le développement, la croissance et la régulation de la fonction reproductrice masculine, par sa capacité à perturber l'interaction du récepteur avec ses régulateurs négatifs, PIASxα (« protein inhibitor of activated STAT ») et DJBP (« DJ-1 binding protein ») (Niki et al., 2003; Takahashi et al., 2001). DJ-1 est aussi capable d'interagir directement avec le récepteur aux androgènes et de réguler son activité transcriptionnelle (Pitkanen-Arsiola et al., 2006; Tillman et al., 2007).

Rôle de DJ-1 dans l'apoptose : Le rôle de DJ-1 dans le contrôle de la mort cellulaire est supporté par plusieurs études. Tout d'abord DJ-1 a été décrite comme régulant p53 et PTEN deux modulateurs clés des voies apoptotiques (Cantley and

Neel, 1999; Kim et al., 2005a; Shinbo et al., 2005). La diminution de l'expression de DJ-1 par différentes techniques (siRNA, invalidation) conduit à une augmentation de la sensibilité aux stress oxydatif et réticulaire (Kim et al., 2005b; Taira et al., 2004; Yokota et al., 2003). La protéine DJ-1 sauvage est capable de restaurer le phénotype induit par l'injection de 6-hydroxydopamine une neurotoxine chez des rats (la mort des cellules dopaminergiques, une diminution du niveau de dopamine et du transporteur de la dopamine associé à un déficit moteur), cette capacité est abolie par la mutation L166P (Inden et al., 2006). La fonction protectrice de DJ-1 semble dépendre de sa capacité à éliminer le peroxyde d'hydrogène *in vitro* par auto oxydation. De fait, la transfection dans des SH-SY5Y du mutant L166P ou de siRNA dirigés contre la protéine DJ-1 sauvage entraîne une augmentation de la sensibilité des cellules à la mort cellulaire induite par différentes molécules oxydantes (H_2O_2, MPP^+, 6-hydroxydopamine) ainsi qu'une diminution des formes oxydées de DJ-1 (Taira et al., 2004; Yokota et al., 2003). De plus, l'oxydation de DJ-1 induit sa relocalisation à la mitochondrie ainsi qu'un rôle protecteur. La relocalisation est abolie par la mutation de la cystéine 106 en alanine, ainsi l'oxydation de la cystéine 106 en acide cystéine sulfonique est essentielle à la fonction protectrice de DJ-1 (Canet-Aviles et al., 2004).

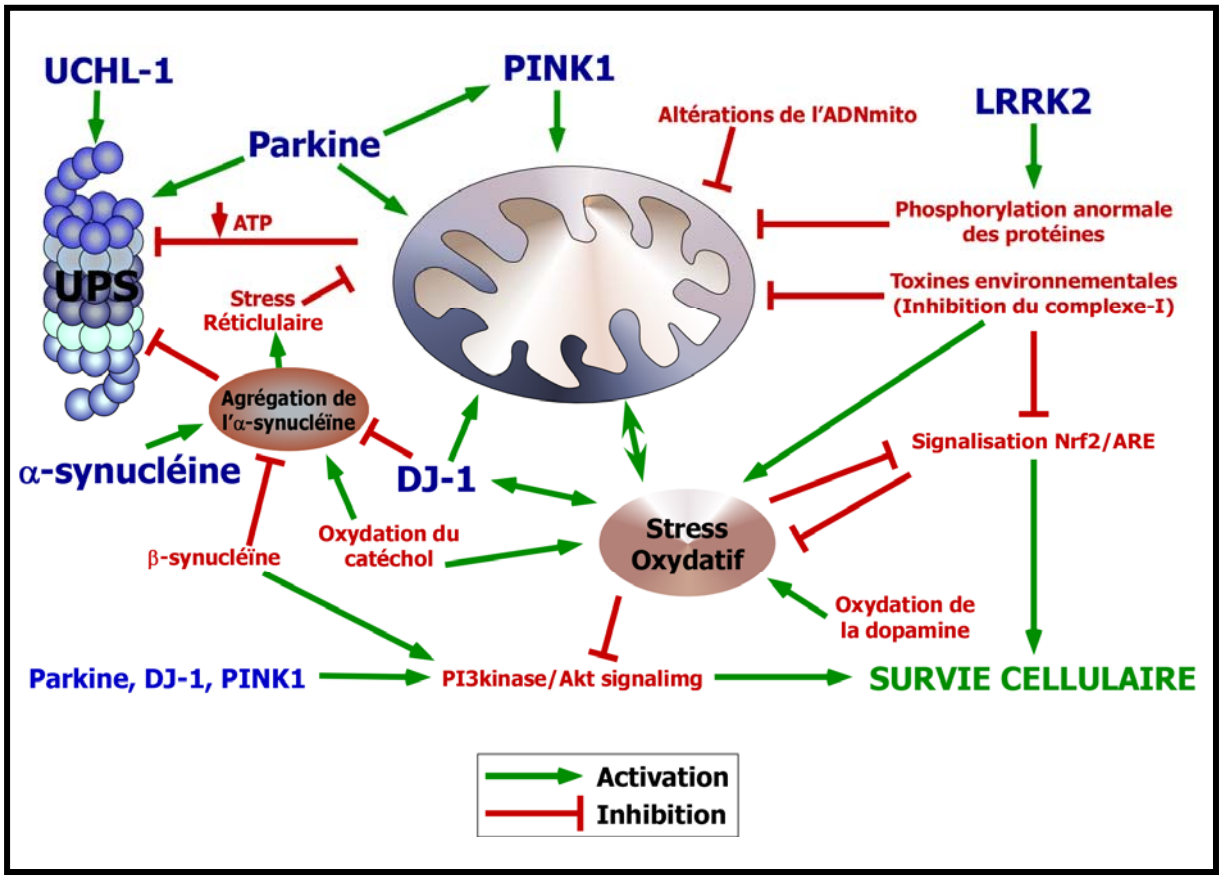
DJ-1 est également capable de contrôler la mort cellulaire par d'autres mécanismes, par exemple, en interagissant avec Daxx, une protéine adaptatrice impliquée dans la voie du récepteur de mort Fas (Junn et al., 2005; Yang et al., 1997). DJ-1 empêche la translocation de Daxx dans le cytoplasme en le séquestrant dans le noyau, empêchant son interaction avec la kinase ASK1 et bloquant ainsi l'activation de cette kinase impliquée dans la mort cellulaire (Charette et al., 2001; Ko et al., 2001; Song and Lee, 2003).

DJ-1 et le stress oxydatif : Mitsumoto et collaborateurs ont démontré en condition de stress oxydatif (exposition à des ROS), un déplacement du point isoélectrique (pI) de DJ-1 de 6,2 vers 5,8, propriété physique des senseurs de stress. DJ-1 pourrait donc se comporter comme une HPRPs (hydroperoxide-responsive proteins) et fonctionner comme un indicateur du stress oxydatif (Mitsumoto and

Nakagawa, 2001). De plus, la spectrométrie de masse a permis de confirmer l'oxydation de DJ-1, ainsi qu'un déplacement de son pI et la formation d'acide cystéine sulfinique. La formation d'acide cystéine sulfinique est une modification post-transcriptionnelle importante associée aux protéines liées au stress oxydatif comme les peroxiredoxines (Kinumi et al., 2004). Des études ont montrées que DJ-1 est oxydé sur la cystéine en position 106. La mutation de cette cystéine abolie sa translocation à la mitochondrie induite par l'oxydation ainsi que ses propriétés anti-apoptotiques (Canet-Aviles et al., 2004). Les mutations pathogènes réduisent également les propriétés anti-oxydantes de DJ-1 (Taira et al., 2004; Takahashi-Niki et al., 2004; Yokota et al., 2003).

Protéine chaperonne : L'homologie de séquence existant entre DJ-1 et les protéines HSP, ainsi que sa présence au sein d'un large complexe moléculaire (>2000kDa) dans des cerveaux en conditions normales et en conditions pathologiques, ont conduit à envisager que DJ-1 pourrait avoir un rôle de chaperonne (Meulener et al., 2005b). Cette fonction de chaperonne a été démontrée *in vitro* par différentes techniques, et semble dépendante des propriétés redox de DJ-1 (Lee et al., 2003; Shendelman et al., 2004). En effet, ce phénotype est abolie lorsque la cystéine en position 53 dans la séquence de DJ-1 est mutée ou lorsque la protéine est pré-incubée avec du DTT empêchant l'oxydation de DJ-1. Cependant une oxydation excessive de DJ-1 induit une perte de son activité de chaperonne : l'inhibition de l'agrégation de l'α-synucléine par DJ-1 est abolie (Meulener et al., 2005b; Zhou et al., 2006). L'activité de chaperonne tout comme la capacité de protéger les cellules, sont dépendantes de la fonction de senseur du stress oxydatif de DJ-1.

Régulation de la transcription : Comme on l'a vu précédemment, DJ-1 est capable de réguler la transcription de plusieurs gènes. En effet, DJ-1 peut par exemple transactiver le récepteur aux androgènes (Niki et al., 2003; Takahashi et al., 2001), p53 (Shinbo et al., 2005) ou transréprimer PTEN (Kim et al., 2005a) et PSF (Xu et al., 2005). L'équipe du Dr Nishinaga a mis en évidence une autre cible de DJ-1 le gène SOD3 (« extracellular superoxide dismutase 3»), compatible avec sa fonction dans la réponse au stress oxydatif. Cette étude a également montré une régulation du



D'après Thomas B. and Beal F., Hum. Mol. Genetics (2007) Vol. 16

Figure 36 : Relations entre les différentes protéines impliquées dans la maladie de Parkinson et les différents systèmes

Grâce à toutes les études menées jusqu'à se jour on peut regrouper toutes les protéines liées aux formes récessives de la maladie sur un même schéma. Il apparait de plus en plus qu'il existe des liens et des voies entre toutes ces protéines.

taux d'ARNm et du promoteur de Tau (protéine majeur dans la pathologie de la maladie d'Alzheimer) (Nishinaga et al., 2005).

DJ-1 possède donc plusieurs fonctions avérées et intervient dans de nombreux processus cellulaires comme le confirment différents travaux effectués dans des souris déficientes pour le gène de DJ-1. En effet, ces souris présentent des déficits moteurs liés à l'âge, des dysfonctions dopaminergiques mais sans perte neuronale (Chen et al., 2005; Goldberg et al., 2005). Kim et collaborateurs ont également observé une augmentation de la sensibilité au stress oxydatif induit par le MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyrindine) ((Kim et al., 2005b), sensibilité qui pourrait être due à l'augmentation de l'expression de p53 et de BAX (Bretaud et al., 2007), ou à un déficit dans la signalisation PI3kinase/Akt (Yang et al., 2005).

DJ-1 a également la capacité d'interagir avec d'autres protéines impliquées dans les formes familiales de la maladie, ce qui en fait une protéine importante dans l'étude des mécanismes liés au développement de la maladie de Parkinson.

A.VI.2.e) Interactions entre les différentes protéines impliquées dans les formes familiales de la maladie:

Comme on a pu le voir jusqu'à présent, certaines des protéines impliquées dans les formes familiales de la maladie de Parkinson peuvent interagir entre elles. C'est le cas pour l'α-synucléine et la synphiline-1 qui interagissent physiquement entre elles. Cette interaction peut se traduire par la formation d'inclusions lorsque ces protéines sont surexprimées ensembles. Des travaux ont également montré une interaction entre la parkine et la synphiline-1 qui conduit à l'ubiquitinylation de cette dernière. De nombreux travaux ont montré que DJ-1 participe aussi à ce réseau (Figure 36).

DJ-1 et l'α-synucléine : DJ-1 et l'α-synucléine n'interagissent pas physiquement dans des cellules provenant de neuroblastomes. Cependant, la surexpression de DJ-1 sauvage inhibe l'accumulation d'α-synucléine dans ces mêmes cellules si elles sont soumises à un prétraitement au $FeCl_2$ (Shendelman et al., 2004), l'interaction entre ces deux protéines dépend donc de l'oxydation de DJ-1 et de son activation en tant que chaperonne. Des études histologiques ont montré la co-localisation et l'interaction de ces deux protéines dans des cerveaux de patients (Meulener et al., 2005b).

DJ-1 et la parkine : Contrairement à ce que l'on attendrait la parkine ne favorise pas la dégradation de mutants L166P et M26I de DJ-1, mais au contraire stabilise ces mutants dans des cultures cellulaires. La protéine DJ-1 sauvage n'interagit pas avec la parkine en condition normale, cependant en condition de stress oxydatif on voit une augmentation de l'interaction entre DJ-1 et la parkine de façon dose dépendante. Dans des cerveaux de patients, l'absence de parkine dans le cortex frontal conduit à une diminution drastique du taux de DJ-1 dans les fractions insolubles, contrairement aux formes sporadiques dans lesquelles on observe une forte augmentation des formes insolubles de DJ-1 (Moore et al., 2005b). La parkine et DJ-1 pourraient intervenir dans les mêmes voies moléculaires impliquées dans la pathogenèse de la maladie de Parkinson.

DJ-1 et PINK1 : Récemment Tang et collaborateurs ont décrit le cas d'une famille dont certains membres présentaient deux mutations hétérozygotes une sur PINK1 (P399L) (une autre protéine impliquée dans les formes récessives de la maladie) et l'autres sur DJ-1 (A39S). Lorsqu'elles sont surexprimées dans des cellules provenant de neuroblastomes les SH-SY5Y ces deux protéines mutées interagissent entre elles et sont localisées à la mitochondrie. De plus elles protègent les cellules de la mort en cas de stress induit par du MPP^+ (Tang et al., 2006).

A.VI) Les modèles de maladie de Parkinson :

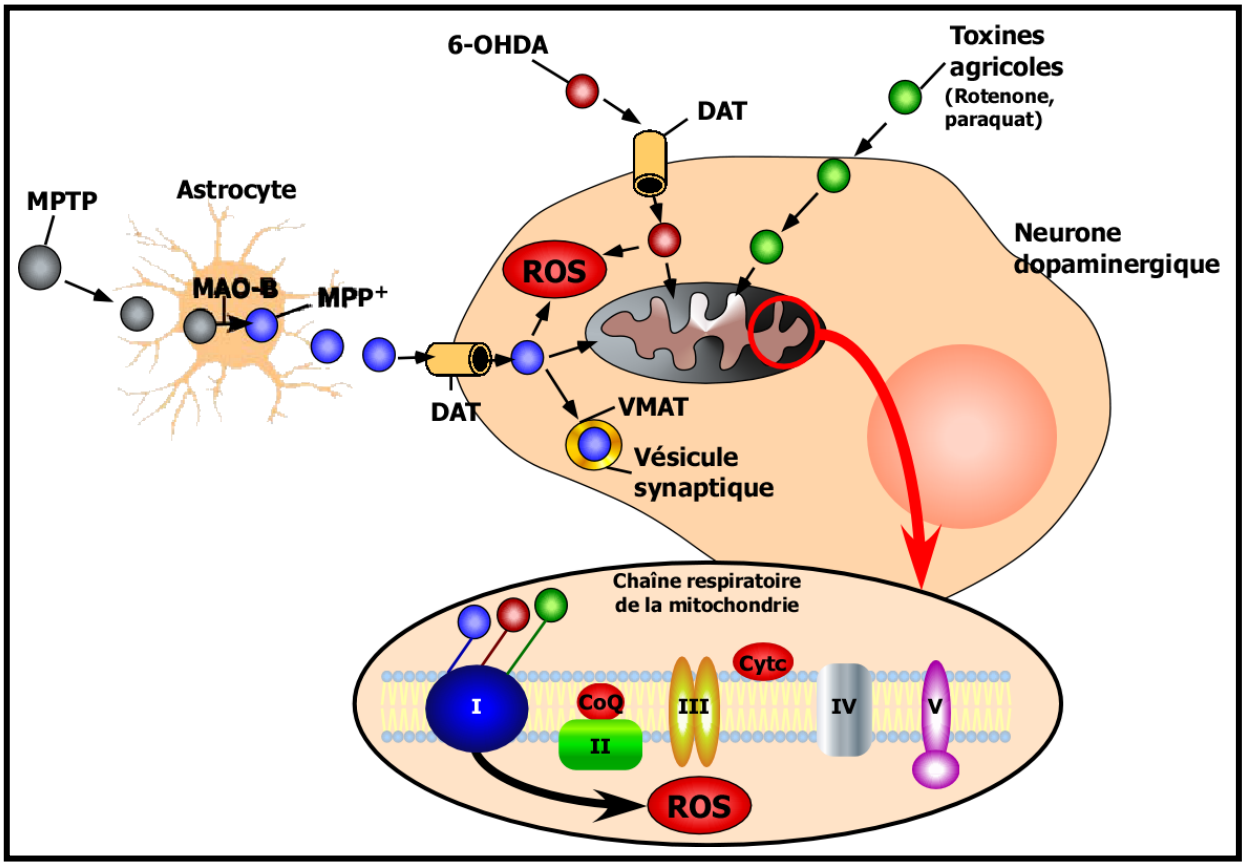
Afin de mieux comprendre les mécanismes impliqués dans le développement de la maladie de Parkinson et parce que cette maladie est inconnue chez la majorité des espèces animales, excepté chez les primates âgés en captivité, il a été nécessaire de développer des modèles animaux. Il existe deux types de modèles : les modèles induits par des toxines, et les modèles liés aux formes génétiques de la maladie.

A.VI.1) Les modèles induits par des toxines :

Actuellement, il existe plusieurs molécules utilisées pour développer des modèles de maladie de Parkinson.

A.VI.1.a) Les modèles induits par la 6-hydroxydopamine :

La 6-hydroxydopamine (6-OHDA) est un analogue hydroxylé naturel de la dopamine (Blum et al., 2001). De façon intéressante cette molécule s'accumule chez les patients parkinsoniens (Andrew et al., 1993). Elle est l'une des molécules les plus utilisée dans les modèles de dégénérescence des projections catécholaminergiques, incluant le système nigro-striatal, *in vitro* et *in vivo* (Blum et al., 2001; Ungerstedt, 1968, 1976). La toxicité induite par la 6-OHDA est spécifique aux neurones catécholaminergiques car elle est essentiellement transportée par les transporteurs dopaminergiques (DAT) et noradrénergiques (Luthman et al., 1989). La 6-OHDA s'accumule à l'intérieur des neurones et provoque leur mort en induisant des mécanismes d'apoptose (Jeon et al., 1995). En effet, la 6-OHDA provoque l'élévation du stress oxydatif par deux processus. D'une part, elle favorise la fabrication de



D'après Schober A., Cell Tissue Res (2004) Vol. 318

Figure 37 : Mécanisme d'action des neurotoxines dopaminergiques

Les trois principales molécules utilisées dans les modèles animaux liés aux toxines vont inhiber le complexe 1 de la chaîne respiratoire. Le MPP+ et la 6-OHDA sont eux spécifiques du transporteur de la dopamine (DAT) donc spécifiques des neurones dopaminergiques.

peroxyde d'hydrogène et de radicaux hydroxyles, ces derniers étant facilement oxydables en présence de fer (Sachs and Jonsson, 1975). D'autre part, elle inhibe le complexe-I de la chaîne respiratoire de la mitochondrie conduisant également à la fabrication de ROS (Figure 37). Classiquement, la 6-OHDA est injectée stéréotaxiquement car lors d'une administration systémique, la 6-OHDA ne parvient pas à passer la barrière hémato-encéphalique. L'injection de 6-OHDA induit spécifiquement la mort des neurones dopaminergiques mais pas la formation d'inclusions cytoplasmiques, ce qui constitue une des limites du modèle. De plus, elle n'affecte pas les aires cérébrales lésées dans le cas d'une maladie de Parkinson comme le bulbe olfactif, ou le locus coeruleus (Betarbet et al., 2002; Del Tredici et al., 2002), mais peut induire l'apparition d'akinésie, de rigidité et de tremblements (Cenci et al., 2002; Lindner et al., 1999) et être utilisée dans des modèles de dyskinésie.

A.VI.1.b) Les modèles induits par le MPTP :

Le 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), neurotoxine dopaminergique a été découvert en 1982 accidentellement. Il s'agit d'un analogue d'un narcotique : la mérépidine (Demerole) (Langston and Ballard, 1983). De jeunes toxicomanes ont développé un syndrome parkinsonien idiopathique après l'administration « d'héroïne synthétique » (MPPP, 1-methyl-4-phenyl-propion-oxypiperidine) (Davis et al., 1979; Langston and Ballard, 1983). Actuellement, cette molécule est la plus fréquemment utilisée dans les modèles animaux. Elle présente d'énormes avantages : elle induit la mort directe et spécifique des structures dopaminergiques, et des symptômes parkinsoniens (Przedborski and Vila, 2003), mais pas la formation d'inclusions cytoplasmiques (Langston and Ballard, 1983). De plus, le MPTP est très lipophilique et passe rapidement la barrière hémato-encéphalique après une administration systémique. En fait, le MPTP est une pro-toxine qui est convertie en 1-methyl-4-phenyl-2,3-dihydropyridium (MPDP) par la monoamine oxydase B (MAO-B) dans les astrocytes et les neurones

sérotoninergiques, MPDP qui est spontanément oxydé en 1-methyl-4-phenylpyridinium (MPP^+) (Nicklas et al., 1985; Nicklas et al., 1987; Przedborski and Vila, 2003). Le MPP^+ va entrer dans la cellule en utilisant le transporteur à la dopamine mais également ceux de la noradrénaline et de la sérotonine (Javitch et al., 1985; Javitch and Snyder, 1984; Mayer et al., 1986). Ces transporteurs sont essentiels puisqu'une souris invalidée pour ces protéines résiste à la toxicité induite par le MPTP (Bezard et al., 1999). Dans les neurones dopaminergiques, le MPP^+ va être incorporé aux vésicules contenant la dopamine en interagissant avec le transporteur vésiculaire de monoamine (VMAT) (Del Zompo et al., 1993), puis va s'accumuler à la mitochondrie et inhiber le complexe-I de la chaîne respiratoire (Hasegawa et al., 1990; Mizuno et al., 1987; Nicklas et al., 1985) induisant ainsi la production de ROS. Le MPP+ peut aussi rester dans le cytoplasme et se lier à différentes enzymes ce qui induit également une augmentation du stress oxydatif (Adams et al., 1993; Klaidman et al., 1993; Ramsay and Singer, 1986). Ces mécanismes conduisent à une mort des neurones par apoptose (Chu et al., 2005) (Figure 37). Toutes les caractéristiques du MPTP en font donc une molécule essentielle dans l'étude de la maladie de Parkinson, mais sa toxicité en fait une molécule dangereuse à manipuler.

A.VI.1.c) Les modèles induits par les pesticides et l'epoxomicine :

Récemment, des agents induisant une toxicité générale ont été utilisés afin de développer des modèles animaux.

On peut citer comme exemple le paraquat, un herbicide ayant une structure similaire à celle du MPTP. Le paraquat n'a pas de sélectivité pour le DAT comme le MPTP, ne s'accumule pas dans les neurones dopaminergiques après des injections systémiques, et passe difficilement la barrière hémato-encéphalique (Shimizu et al., 2001). Cependant cette molécule entraine la mort des neurones dopaminergiques de la substance noire (McCormack et al., 2002; Ossowska et al., 2006) (Figure 37). La

roténone, un insecticide produit à partir de racines et de tiges de plantes tropicales et inhibant le transfert d'électrons entre le complexe-I et l'ubiquinone dans la chaîne respiratoire mitochondriale est également utilisée. La roténone agit sur les mêmes sites que le MPTP mais n'a qu'une toxicité moyenne pour l'homme et est très instable (demi-vie très courte dans l'environnement) (Figure 37). Dans des modèles animaux, en particulier chez le rat, elle induit une lente dégénérescence des neurones dopaminergiques associée à la formation d'inclusions intra-cytoplasmiques contenant de l'α-synucléine, une modification de l'état d'oxydation de DJ-1 et des dysfonctionnements du protéasome (Betarbet et al., 2006). Malheureusement, les modèles utilisant la roténone ont une faible reproductibilité et de nombreux animaux meurent du fait de sa toxicité non liée au système nerveux central (Talpade et al., 2000). Pour terminer, l'administration systémique d'un inhibiteur du protéasome l'epoxomicine a récemment été utilisée pour développer un modèle de la maladie de Parkinson chez le rat (McNaught et al., 2004). Ce modèle regroupe les caractéristiques clé de la pathologie : réduction des fibres dopaminergiques dans le striatum, dégénérescence des neurones dopaminergiques accompagnée d'une inflammation et d'agrégats intracellulaires contenant de l'α-synucléine et de l'ubiquitine. Cependant Kodower et collaborateurs n'ont pu reproduire ces observations chez les rats et les singes (Kordower et al., 2006).

A.VI.2) Les modèles transgéniques :

Actuellement plusieurs modèles génétiques existent et ceci dans différentes espèces : Souris, Drosophile et Caenorhabditis elegans. Je me focaliserai ici uniquement sur les modèles génétiques liés aux protéines auxquelles je me suis intéressée tout au long de ce manuscrit (α-synucléine, DJ-1 et parkine).

A.VI.2.a) Les modèles liés à l'α-synucléine :

En complément des modèles de souris déficientes pour le gène de l'α-synucléine qui ont permis d'identifier l'α-synucléine comme modulateur négatif de la neurotransmission dopaminergique (Abeliovich et al., 2000) : ces souris sont viables, fertiles, ont une architecture cérébrale normale, elles sont cependant sujettes à une exacerbation de la libération de dopamine en conditions stimulées. Depuis ces 10 dernières années de nombreuses générations de souris et de drosophiles transgéniques pour l'α-synucléine sauvage ou mutée ont vu le jour (Feany and Bender, 2000; Giasson et al., 2002; Lee et al., 2002c; Masliah et al., 2000; Matsuoka et al., 2001; Richfield et al., 2002; van der Putten et al., 2000).

Ces souris montrent divers changements neuropathologiques incluant une atrophie neuronale, une dystrophie des neurites et des astrocytes, accompagnées d'inclusions semblables aux corps de Lewy contenant de l'α-synucléine. Cependant ces animaux ne présentent aucun défaut au niveau des neurones dopaminergiques, ce qui constitue une limite de ce modèle (Giasson et al., 2002; Lee et al., 2002c; Matsuoka et al., 2001).

Les modèles transgéniques α-synucléine dans les drosophiles possèdent le phénotype complet de maladie de Parkinson, incluant la perte des neurones dopaminergiques, des inclusions intracytoplasmiques positives pour l'α-synucléine, ainsi que des troubles moteurs. Le phénotype observé chez les drosophiles transgéniques pour l' α-synucléine en font un modèle particulièrement intéressant pour l'étude de maladie de Parkinson (Bilen and Bonini, 2005; Feany and Bender, 2000; Pendleton et al., 2002).

Il existe également des modèles transgéniques chez C. elegans qui développent des troubles moteurs et une perte neuronale. Ces modèles pourraient faciliter la dissection des mécanismes physiopathologiques liés à la maladie de Parkinson du fait de la simplicité de l'organisme (Lakso et al., 2003; Vartiainen et al., 2006).

Récemment, deux groupes ont développé des modèles de maladie de Parkinson dans chez des rats adultes. Ces modèles sont produits en injectant des vecteurs viraux exprimant l'α-synucléine humaine dans la substance noire. Ces injections entrainent la mort des neurones dopaminergiques, et la formation d'inclusions cytoplasmique contenant de l'α-synucléine. Grâce à l'utilisation de vecteurs viraux il existe maintenant des modèles complets de maladie de Parkinson chez les mammifères induit par l'α-synucléine (Kirik et al., 2002; Lo Bianco et al., 2002).

A.VI.2.b) Les modèles liés à la parkine :

Actuellement il n'existe pas de souris transgénique surexprimant le gène humain de la parkine, il existe cependant des modèles d'invalidation du gène de la parkine. Ces souris ont une altération du système dopaminergique, une diminution de l'activité de la chaîne respiratoire et donc une augmentation du stress oxydatif, ainsi qu'une diminution de la capacité motrice (Goldberg et al., 2003; Palacino et al., 2004). Très récemment un modèle transgénique chez la drosophile a été généré, ces mouches présentent une perte des neurones dopaminergiques, accompagnée d'une perte des capacités motrices (Sang et al., 2007).

A.VI.c) Les modèles liés à DJ-1 :

Tout comme pour la parkine il n'existe pas à l'heure actuelle de souris transgénique surexprimant le gène de DJ-1, mais il existe des souris invalidées pour ce gène. Les souris sont viables, fertiles et n'ont pas d'anomalies anatomiques neuronales majeures. Ces souris présentent une augmentation du taux de transporteurs de la dopamine, ce qui pourrait induire une augmentation de la toxicité liée à la dopamine dans les neurones. De fait, ces souris développent une hypersensibilité au stress oxydatif, une exposition au MPTP induit une perte des

capacités locomotrices (Chen et al., 2005; Goldberg et al., 2005; Kim et al., 2005b; Manning-Bog et al., 2007; Yamaguchi and Shen, 2007). On retrouve également ce phénotype chez les drosophiles invalidées pour le gène de DJ-1 (Meulener et al., 2005a; Meulener et al., 2006; Park et al., 2005; Yang et al., 2005).

Tous ces modèles permettent de disséquer les différents mécanismes impliqués dans la pathogénèse de la maladie de Parkinson, aussi bien les mécanismes généraux (augmentation du stress oxydatif, apoptose), que l'implication directe de diverses protéines. La combinaison de différents modèles (toxines et génétiques) améliore encore la compréhension des processus liés à cette maladie.

B) Résultats

B.I) Modulation de l'apoptose dépendante de p53 par le fragment C-terminal de la synphiline-1 (Article 1)

Article 1: "Caspase-3-derived C-terminal product of synphilin-1displays antiapoptotic function via modulation of the p53-dependent cell death pathway." The Journal of Biological Chemistry, 2006, 281, (17), 11515-11522.

Lorsque je suis arrivée au laboratoire et que j'ai commencé cette étude sur la synphiline-1 très peu de choses étaient connues à son sujet, en effet, cela faisait peu de temps qu'elle avait été découverte. Au laboratoire, le Dr Alves da Costa avait mis en évidence que l'α-synucléine joue un rôle protecteur dans l'apoptose et que cette fonction était abolie non seulement par les mutations pathogènes mais aussi par la 6-hydroxydopamine (Alves da Costa et al., 2000; Alves da Costa et al., 2006; Alves da Costa et al., 2002). Le rôle de l'α-synucléine dans les processus apoptotiques nous a conduits à nous interroger sur la fonction putative de la synphiline-1 un de ses partenaires dans ces processus.

La synphiline-1 n'est pas sensible aux métalloprotéases, aux protéases acides et aux calpaïnes.

Nous nous sommes tout d'abord intéressés à la stabilité de cette protéine. Le préalable à cette étude était l'établissement de deux lignées cellulaires de différentes origines une rénales ("human embryonnic kidney" : HEK293) et une neuronale ("Telencephalon specific mouse 1" :TSM1) exprimant stablement la synphiline-1 sauvage et la synphiline-1 mutée an position 454 où un aspartate a été muté alanine (Figure 1 et Figure 6A). Afin de savoir si la synphiline-1 pouvait subir un ou plusieurs clivages et quelles classes de protéases pouvaient être impliquées, nous avons traité ces lignées cellulaires avec la pepstatine (inhibiteur des protéases acides), l'ALLN (inhibiteur), ou encore l'O-phénanthroline (pour les métalloprotéases). Ces inhibiteurs n'ont pas d'effet protecteur sur l'expression de la synphiline-1 (Figure 2).

La synphiline-1 est clivée par une caspase effectrice.

Lors d'un traitement de ces lignées avec l'Ac-DEVD-CHO (inhibiteur des caspase-3, -6 et -7) ou l'E64 (ciblant les protéases à thiol), on observe une augmentation de l'expression de la synphiline-1 sauvage. De façon intéressante le mutant D454A est insensible à cet inhibiteur (Figure 2). Nous avons donc recherché la présence de sites spécifiques des caspases, cela nous a conduits à identifier un site de clivage putatif de la caspase-3, 451DEVD454. Afin de déterminer si cette protéine pouvait être clivée, nous avons utilisé une technique de transcription et de traduction *in vitro* à l'aide d'un lysat de réticulocytes de lapin permettant de synthétiser les protéines. Nous avons ensuite incubé les protéines produites en présence de caspases-3, -6 et -7 recombinantes.

Cette expérience nous a permis de confirmer le clivage de la synphiline-1 par les caspases effectrices mais surtout d'identifier la caspase-3 comme étant la caspase responsable de ce clivage, de plus le mutant D454A de la synphiline-1 est résistant au clivage par la caspase-3, nous avons donc bien muté un site caspase (Figure 3).

Les cellules surexprimant la synphiline-1 sauvage sont moins sensibles à l'apoptose

Les cellules HEK293, TMS1, ou SH-SY5Y (des cellules issues de neuroblastomes humains possédant toute la machinerie dopaminergique) surexprimant stablement ou transitoirement la synphiline-1 sauvage sont plus résistantes à l'apoptose induite par la staurosporine (STS), un inhibiteur de la protéine kinase C et surtout par la 6-OHDA. En effet, dans ces cellules la surexpression de la synphiline-1 sauvage induit une diminution de l'activation des caspases effectrices. Cette activité est mesurable par un dosage fluorométrique, mais également visible lorsque l'on suit l'expression de PARP (poly (ADP-ribose) polymérase) une enzyme de réparation de l'ADN substrat des caspases, ainsi que l'expression du précurseur inactif des caspases (Pro-CPP32). De façon intéressante, la mutation D454A sur la synphiline-1 abolit cette fonction protectrice (Figure 4, 6 et 7).

La synphiline-1 sauvage mais pas la synphiline-1 D454A régule p53.

Dans cette étude nous avons déterminé que la fonction protectrice de la synphiline-1 passe par la régulation de la voie dépendante du facteur de transcription p53, en effet, la synphiline-1 est capable de réguler négativement l'expression nucléaire et l'activité de p53. Nous avons voulu déterminer à quel niveau la synphiline-1 pouvait moduler p53 ; nous avons donc vérifié l'effet de la synphiline-1 sur la transactivation du promoteur de p53 ainsi que son influence sur le taux d'ARN de p53. Tout comme pour l'expression et l'activité de p53, nous avons observé une diminution de la transactivation de son promoteur et de son niveau d'ARN messager, de façon intéressante, la synphiline-1 mutée n'est pas capable de réguler négativement la voie dépendante de p53 (Figure 5).

L'activité protectrice de la synphiline-1 est dépendante de son fragment C-terminal.

Le fait que la synphiline-1 mutée soit incapable de protéger les cellules de l'apoptose nous a conduits à nous intéresser au rôle du fragment C-terminal issu du clivage de la synphiline-1 par la caspase-3. Nous avons synthétisé le fragment C-terminal issu de ce clivage étiqueté avec le tag V5. Ce fragment, tout comme la synphiline-1 sauvage, est capable de diminuer l'activité des caspases effectrices induite par la STS et la 6-OHDA lorsqu'il est transfecté transitoirement dans des HEK293 (Figure 8).

Conclusion/ Discussion.

Ce travail nous a permis de déterminer une nouvelle fonction de la synphiline-1, une nouvelle protéine dont la ou les fonctions restent encore mal connues. Cette protéine exerce, en effet, une fonction protectrice qui passe par

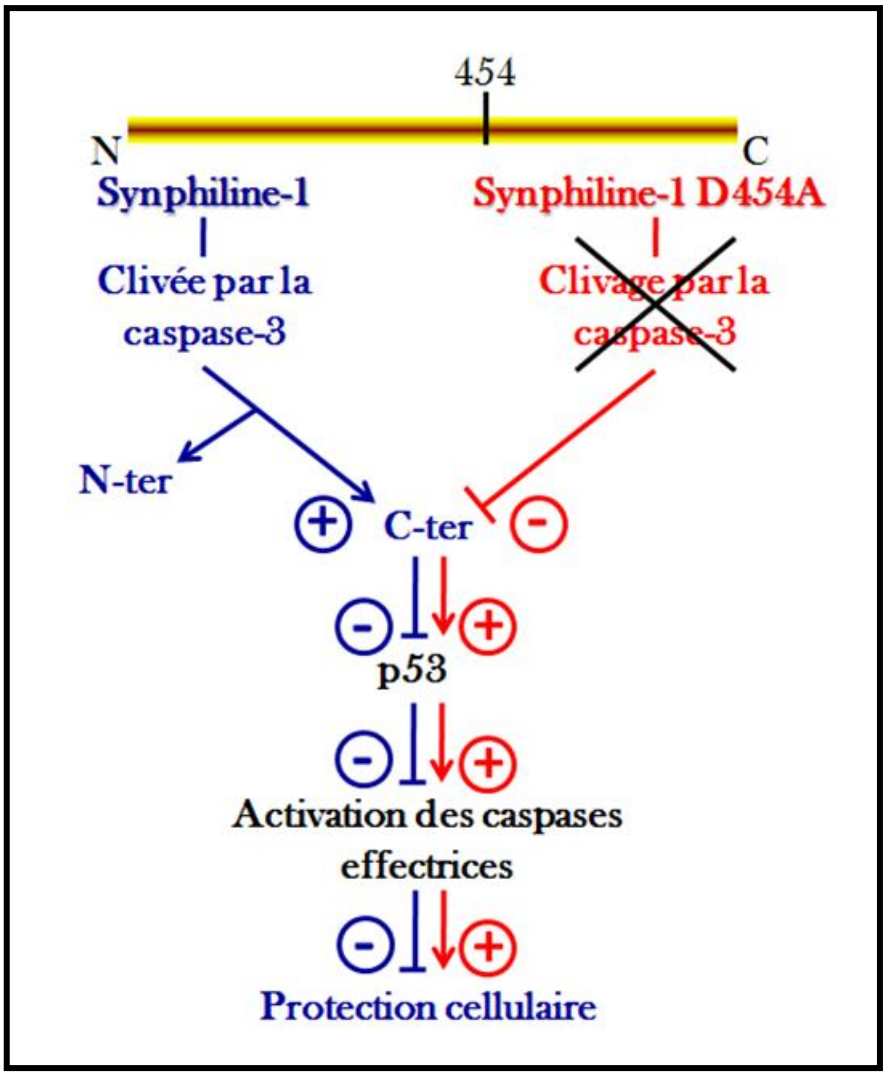


Figure 38 : Schéma récapitulant les mécanismes impliqués dans la fonction protectrice de la synphiline-1

Cette figure regroupe tous les mécanismes et les intermédiaires impliqués dans la fonction protectrice de la synphiline-1 que j'ai identifié dans ce travail.

l'inhibition de la voie pro-apoptotique p53, fonction qui est abolie par la mutation D454A au niveau du site de clivage de la caspase-3, cette fonction semble assurée par le fragment C-terminal de la synphiline-1 issue de son clivage par la caspase-3 (Figure 38). Toutes les protéines impliquées dans les formes familiales de la maladie sont plus ou moins liées entre elles. En effet, l'α-synucléine interagit également avec la parkine, l'UCHL1 avec la mortaline, la nucleoline et la calnexine, trois protéines qui interagissent également avec DJ-1 (Jin et al., 2007). Il serait intéressant de savoir si l'interaction physique existant entre la synphiline-1 et l'α-synucléine se traduit par une interaction phénotypique. En outre, la synphiline-1 interagissant avec la parkine (Chung et al., 2001), il sera intéressant de savoir si elle est également capable d'interagir avec d'autres protéines impliquées dans les formes familiales physiquement ou fonctionnellement. En dernier lieu, il faudra envisager de déterminer l'influence du clivage de la synphiline-1 sur les fonctions de l'α-synucléine et de la parkine, deux partenaires connus mais également sur ceux encore inconnus à ce jour.

Article 1

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Caspase-3-derived C-terminal Product of Synphilin-1 Displays Antiapoptotic Function via Modulation of the p53-dependent Cell Death Pathway*

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Parkinson disease is the second most frequent neurodegenerative disorder after Alzheimer disease. A subset of genetic forms of Parkinson disease has been attributed to α-synuclein, a synaptic protein with remarkable chaperone properties. Synphilin-1 is a cytoplasmic protein that has been identified as a partner of α-synuclein (Engelender, S., Kaminsky, Z., Guo, X., Sharp, A. H., Amaravi, R. K., Kleiderlein, J. J., Margolis, R. L., Troncoso, J. C., Lanahan, A. A., Worley, P. F., Dawson, V. L., Dawson, T. M., and Ross, C. A. (1999) *Nat. Gen.* 22, 110–114), but its function remains totally unknown. We show here for the first time that synphilin-1 displays an antiapoptotic function in the control of cell death. We have established transient and stable transfectants overexpressing wild-type synphilin-1 in human embryonic kidney 293 cells, telecephalon-specific murine 1 neurons, and SH-SY5Y neuroblastoma cells, and we show that both cell systems display lower responsiveness to staurosporine and 6-hydroxydopamine. Thus, synphilin-1 reduces procaspase-3 hydrolysis and thereby caspase-3 activity and decreases poly(ADP-ribose) polymerase cleavage, two main indicators of apoptotic cell death. Furthermore, we establish that synphilin-1 drastically reduces p53 transcriptional activity and expression and lowers p53 promoter transactivation and mRNA levels. Interestingly, we demonstrate that synphilin-1 catabolism is enhanced by staurosporine and blocked by caspase-3 inhibitors. Accordingly, we show by transcription/translation assay that recombinant caspase-3 and, to a lesser extent, caspase-6 but not caspase-7 hydrolyze synphilin-1. Furthermore, we demonstrate that mutated synphilin-1, in which a consensus caspase-3 target sequence has been disrupted, resists proteolysis by cellular and recombinant caspases and displays drastically reduced antiapoptotic phenotype. We further show that the caspase-3-derived C-terminal fragment of synphilin-1 was probably responsible for the antiapoptotic phenotype elicited by the parent wild-type protein. Altogether, our study is the first demonstration that synphilin-1 harbors a protective function that is controlled by the C-terminal fragment generated by its proteolysis by caspase-3.

Parkinson disease (PD)[3] is characterized by the presence of intracytoplasmic inclusions, named Lewy bodies (LB), and by a massive loss of dopaminergic neurons in the substantia nigra (2). Most PD cases are of sporadic origin, but about 15% are associated with genetic causes. From the various loci associated with PD, named PARKs, six proteins have been identified so far. They are linked to either autosomal dominant (α-synuclein, UCHL1, and LRRK2/dardarin) or autosomal recessive (parkin, DJ-1, and PINK-1) transmission (3–5), the latter forms being more severe and characterized by an early onset. The above proteins are linked to the three major dysfunctions observed in PD, which are oxidative stress, mitochondrial failure, and proteasomal dysfunction (6). Interestingly, these dysfunctions are associated with exacerbated cell death in PD (7–9).

Among the proteins responsible for the familial forms of PD, α-synuclein has received particular attention, not only because it was the first gene product implicated in familial forms of the disease but also because it is the major fibrillar protein of the LB (10, 11). Even if its function is far from being completely elucidated, α-synuclein seems to play a major role in cell death processes. Thus, we have shown that wild-type α-synuclein triggered an antiapoptotic response in TSM1 neurons and that this phenotype could be abolished by familial PD mutations and 6-hydroxydopamine (6OH-DOPA) (12, 13), a natural endogenous dopaminergic toxin (14) that is frequently used to induce PD *in vivo* (15–17).

The hunt for putative physiological binding partners of a protein often gives insight into its function. This strategy has led to the identification of a novel α-synuclein cellular partner named synphilin-1, the function of which remains completely unknown (1). Synphilin-1 is a cytoplasmic protein of 919 amino acids that has been identified by a yeast two-hybrid approach (1). The putative relevance of this protein to PD pathology is emphasized by several studies. Thus, synphilin-1 interacts with two proteins linked to familial PD (*e.g.* α-synuclein and parkin) (1, 18) and is expressed in 80–90% of the LB detected in PD brain samples (19). *In vitro* studies have shown that the co-expression of α-synuclein and synphilin-1 favor the formation of cytoplasmic inclusions that resemble LB *in vivo* (1, 20, 21). Synphilin-1 is located within a region of the chromosome 5q23.1–23.3 that is characterized by evocative lod scores for PD in distinct whole genome scans (22–24). Indeed,

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[3] The abbreviations and trivial name used are: PD, Parkinson disease; LB, Lewy bodies; 6OH-DOPA, 6-hydroxydopamine; Ac-DEVD-CHO or DEVD, acetyl-Asp-Glu-Val-Asp-aldehyde; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; WT-synphilin-1, wild-type synphilin-1; STS, staurosporine; ALLN, *N*-acetyl-L-leucyl-L-norleucinal; E64, L-*trans*-epoxysuccinyleucilamino(*n*-guanino)butane.

mutation analysis of the synphilin-1 gene in familial and sporadic German PD patients allowed the identification of the R621C mutation in two sporadic PD patients, suggesting a putative role of synphilin-1 as a genetic susceptibility factor for the disease (25). Due to the implication of synphilin-1 in PD and to the modulation of cell death by α-synuclein and parkin, two privileged binding partners of synphilin-1, we investigated the role of synphilin-1 in cell death control. We show that synphilin-1 lowers HEK293 cells, TSM1 neurons, and SH-SY5Y neuroblastoma responsiveness to staurosporine and 6OH-DOPA by decreasing caspase-3 activity and poly(ADP-ribose) polymerase and by down-regulating the p53-dependent proapoptotic pathway. In addition, *in silico* examination of the synphilin-1 sequence revealed a consensus site for a caspase-3 cleavage. Accordingly, we demonstrate the cleavage of synphilin-1 by cellular and purified caspase-3 and the abolishment of its antiapoptotic function by site-directed mutagenesis of the caspase-3 site in its sequence. Finally, we demonstrate that the C-terminal fragment of synphilin-1 generated by caspase-3 is indeed responsible for the antiapoptotic phenotype of synphilin-1.

EXPERIMENTAL PROCEDURES

Materials—Lactacystin, acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO; DEVD), pepstatin, L-*trans*-epoxysuccinyleucil-amino-(*n*-guanino) butane (E64), *N*-acetyl-L-leucyl-L-norleucinal (ALLN), *o*-phenanthroline, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, staurosporine, and 6OH-DOPA were purchased from Sigma.

Mutagenesis—A putative consensus cleavage site for caspase-3 was identified in synphilin-1, *in silico*, by means of the peptide cutter ExPAsy software. The D454A-synphilin-1 was obtained by oligonucleotide-directed mutagenesis from wild-type synphilin-1 V5-tagged cDNA by means of a QuikChange™ site-directed mutagenesis kit (Stratagene). The two primers 5′-GGCATCTCGTTGGATGAAGTAGCACAG-GATGGCAAC-3′ and 5′-GTTGCCATCCTGTGCTACTTCATC-CAACGAGATGCC-3′ (Eurogentec) containing the D454A mutation were designed according to the manufacturer's conditions.

The cDNA encoding the V5-tagged caspase-3-derived C-terminal fragment of synphilin-1 was engineered by introducing an ATG codon in position 454 after the putative consensus cleavage site of caspase-3 (oligonucleotide 5′-TA-CCC-AAG-CTT-ATG-CAG-GAT-GGC-3′). An additional HindIII restriction site was also added, adjacent to the ATG codon, for further subcloning of the construction in pcDNA3.1/V5/His-TOPO.

Cell Systems and Transfections—TSM1 neurons (26), HEK293 human cells, and SH-SY5Y neuroblastoma were cultured as previously described (27, 28). Stable transfectants expressing empty vector (mock) and wild-type and mutated synphilin-1 in HEK293 cells were obtained after transfection with 2 μg of each cDNA (all in pcDNA3) by means of calcium phosphate precipitation. TSM1 neurons expressing empty vector (mock) and wild-type synphilin-1 were obtained after the transfection with 2 μg of each cDNA by means of Superfect reactive according to the manufacturer's conditions. Positive clones were screened for their synphilin-1-like immunoreactivity as described below. Transient transfections were carried out by means of 2 μg of cDNA by calcium phosphate precipitation (HEK293 cells) or Lipofectamine (4 μl; TSM1 and SH-SY5Y).

Wild-type and Mutated Synphilin-1 Degradation—Wild-type and D454A-synphilin-1-overexpressing HEK293 and TSM1 cells were preincubated for 16 h in the absence or in the presence of various protease inhibitors at the following concentrations: Ac-DEVD-CHO (100 μM), pepstatin (10 μM), ALLN (100 μM), E64 (100 μM), *o*-phenanthroline (100 μM). Then cells were lysed and analyzed for synphilin-1-like immunoreactivity by Western blot using anti-V5 antibodies as described below.

Western Blot Analysis—For the detection of wild-type and mutated synphilin-1, equal amounts of protein (50 μg) were separated on 8% gels and Western blotted with the anti-V5 mouse monoclonal antibodies (Invitrogen). For the detection of procaspase-3, human and mouse PARP, and β-tubulin immunoreactivities, equal amounts of protein (25 μg) were separated on 8 or 12% gels and Western blotted with anti-human procaspase-3 antibodies (Interchim) and anti-human (Euromedex) and anti-mouse (BD Biosciences) PARP antibodies. Anti-β-tubulin and anti-actin monoclonal antibodies were from Sigma. Immunological complexes were revealed as previously described (29).

Caspase-3 Activity Measurements—Stable transfectants were preincubated without or with staurosporine (0.5–2 μM) or 6OH-DOPA (0.03–0.3 mM) for various times, and then caspase-3-like activity was fluorimetrically measured as extensively detailed (12). Caspase-3-like activity is considered as the Ac-DEVD-CHO-sensitive Ac-DEVD-7-amino-4-methylcoumarin-hydrolyzing activity.

p53 Expression, Activity, and Promoter Transactivation—The activity of p53 was analyzed after transient transfection of the PG13-luciferase (PG13) cDNA designed and kindly provided by Dr. B. Vogelstein (Baltimore, MD) (30). The transcriptional activation of the human p53 promoter (*hpp53*) was measured after transfection of the cDNA coding for the human p53 promoter sequence in frame with luciferase (provided by Dr. M. Oren, Rehovot, Israël). All activities were measured after co-transfection of 0.5–1 μg of the above cDNAs and 0.25–0.5 μg of β-galactosidase cDNA, in order to normalize transfection efficiencies.

p53 immunoreactivity was analyzed by Western blot using an anti-p53 mouse monoclonal antibody (1:10,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in nuclear extracts prepared as previously described for cytochrome *c* translocation experiments (13).

Real Time Quantitative PCR—Total RNA from cells was extracted at the indicated times using the RNeasy kit following the instructions from the manufacturer (Qiagen). After treatment with DNase I, 2 μg of total RNA were reverse transcribed using oligo(DT) priming and avian myeloblastosis virus reverse transcriptase (Promega). Real time PCR was performed in an ABI PRISM 5700 Sequence Detector System (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer. Gene-specific primers were designed using the Primer Express software (Applied Biosystems). Relative expression level of target genes was normalized for RNA concentrations with two different housekeeping genes (human glyceraldehyde-3-phosphate dehydrogenase, mouse γ-actin) according to the cell specificity.

In Vitro Transcription/Translation of Wild-type and Mutated Synphilin-1 and Cleavage by Caspase-3, -6, and -7 in a Cell-free System—Wild-type and D454A synphilin-1 were transcribed and translated using the Promega TNT coupled reticulocyte lysate system in the presence of [^{35}S]methionine (ICN) as extensively described (31). Briefly, 2.5 μl of reticulocyte lysates were incubated in 50 μl of 25 mM HEPES, pH 7.5, 0.1% CHAPS, 5.0 mM dithiothreitol with 25 ng of recombinant caspase-3, -6, and -7 (Sigma) for 8 h at 37 °C. In some experiments, the effect of the caspase inhibitor benzyloxycarbonyl-VAD (10 μM) was examined. Proteins were then electrophoresed on 11% polyacrylamide gels and autoradiographed using Amersham Biosciences hyperfilms.

Statistical Analysis—Statistical analysis was performed with PRISM software (Graphpad Software, San Diego, CA), by using the Newman-Keuls multiple comparison tests for one-way analysis of variance and Student's *t* test.

RESULTS

Wild-type Synphilin-1 (WT-synphilin-1) but Not D454A-synphilin-1 Undergoes Cellular Proteolysis by Ac-DEVD-CHO-sensitive Caspase-like Activity in HEK293 Human Cells and Is Cleaved by Purified Caspase-3 in Vitro—We have established stable transfectants overexpressing WT-synphilin-1 and mutated D454A-synphilin-1 in human embryonic kidney (HEK293) cells. The design of mutated D454A-synphilin-1 is based on an *in silico* study that identified a consensus cleavage site for caspase-3 (^{451}DEVD^{454}) on the WT-synphilin-1 sequence. Fig. 1*A* shows several of the wild-type and mutated stable transfectants obtained that overexpress a 120-kDa protein, a molecular mass corresponding to that expected for the V5-tagged synphilin-1 (1). Clones 10 and 11 (Fig. 1*B*), which display similar levels of wild-type and mutated synphilin-1 protein expression, were selected for the follow-up of our study.

Fig. 2 illustrates the susceptibility of WT-synphilin-1 to various protease inhibitors. Pepstatin (acidic protease inhibitor), ALLN (calpain inhibitor), and *o*-phenanthroline (metalloprotease inhibitor) were unable to affect WT-synphilin-1 expression (Fig. 2, *A* and *B*). Ac-DEVD-CHO (caspase-3, -6, and -7 inhibitor) significantly increased WT-synphilin-1 immunoreactivity (Fig. 2, *A* and *B*) in a time-dependent manner (Fig. 2*C*), suggesting a processing of this protein by caspases. It is interesting to note that E64 (cysteine/serine protease inhibitor) also slightly but significantly potentiated WT-synphilin-1 expression (Fig. 2, *A* and *B*), in agreement with the fact that caspases activities belong to the class of cysteine proteases (32, 33). Interestingly, D454A-synphilin-1 remained completely insensitive to both E64 and caspase inhibitor (Fig. 2, *A* and *C*). These data first confirm that the D454A mutation renders synphilin-1 resistant to proteolysis in HEK293 cells and indicates that caspase-like activities mainly contributed to synphilin-1 catabolism in HEK293 cells.

Staurosporine and 6OH-DOPA have been shown to increase caspase-3 activity in various cell systems (13). We therefore examined whether treatment of WT-synphilin-1-expressing cells with these two proapoptotic effectors could enhance WT-synphilin-1 degradation. Indeed, Fig. 2*D* shows that staurosporine and 6OH-DOPA both decreased the expression of WT-synphilin-1, the levels of which appeared drastically increased upon Ac-DEVD-CHO treatment of the cells (Fig. 2*D*), in agreement with the above data suggesting an implication of caspases in the processing of synphilin-1. In order to identify the caspases involved in the cleavage of synphilin-1, we examined its susceptibility to proteolysis by recombinant caspase-3, -6, and -7 *in vitro*. Fig. 3*A* shows that WT-synphilin-1 is cleaved by recombinant caspases-3 and, to a much lesser extent, by caspase-6, whereas caspase-7 appeared unable to cleave WT-synphilin-1 (Fig. 3*A*). Ac-DEVD-CHO fully prevented caspase-3 and caspase-6-mediated hydrolysis of WT-synphilin-1 (Fig. 3*A*). Interestingly, D454A-synphilin-1 fully resisted proteolysis by recombinant caspase-3 (Fig. 3*B*). It should be noted that WT-synphilin-1 resisted proteolysis by recombinant and cellular overexpressed caspase-8 (not shown), in agreement with the fact that the site cleaved in synphilin-1 (DEVD ↓ Q) and mutated in D454A-synphilin-1 is canonical for caspase-3 but not caspase-8.

WT-synphilin-1 but Not D454A-synphilin-1 Reduces Staurosporine- and 6OH-DOPA-induced Caspase-3 Activation in HEK293 Cells and Lowers the p53-dependent Proapoptotic Pathway—The implication of caspases in the processing of synphilin-1 led us to investigate whether WT-synphilin-1 could control cell death and whether the caspase site mutation could influence such a phenotype. We analyzed the responsiveness of transiently or stably transfected WT-synphilin-1 and D454A-synphilin-1-expressing HEK293 cells to staurosporine (STS) and 6OH-DOPA and, more particularly, the levels of caspase-3. Staurosporine was used as a broad and nonspecific proapoptotic inducer, whereas 6OH-DOPA is a natural dopaminergic toxin that triggers neurodegenerescence that mimics that observed in PD pathology (2). First, we confirmed that STS (Fig. 4, *A*, *C*, and *G*) and

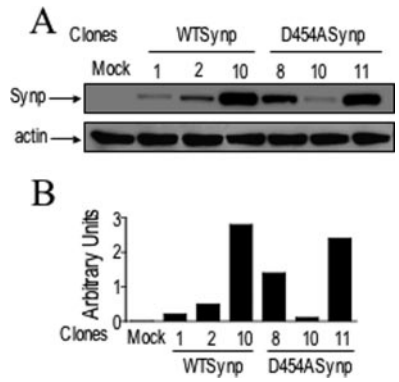

FIGURE 1. **Immunological analysis of WT-synphilin-1- and mutated D454A-synphilin-1-expressing HEK293 cells.** HEK293 cells were stably transfected with empty pcDNA3 vector (*Mock*), wild-type-synphilin-1 (*WTSynp*), or D454A-synphilin-1 (*D454ASynp*) cDNA as described under "Experimental Procedures." Synphilin-1-like immunoreactivities of wild-type (*clones WT*) and mutated synphilin-1 (*clones D454A*) were analyzed by electrophoresis on a 8% Tris-glycine gel, Western Blot, and incubation with anti-V5 primary antibodies as described under "Experimental Procedures" (*A*). Actin immunoreactivity was monitored as a control of protein charge (see "Experimental Procedures"). In *B*, the *bars* correspond to the densitometric analyses of the various clones normalized for actin expression.

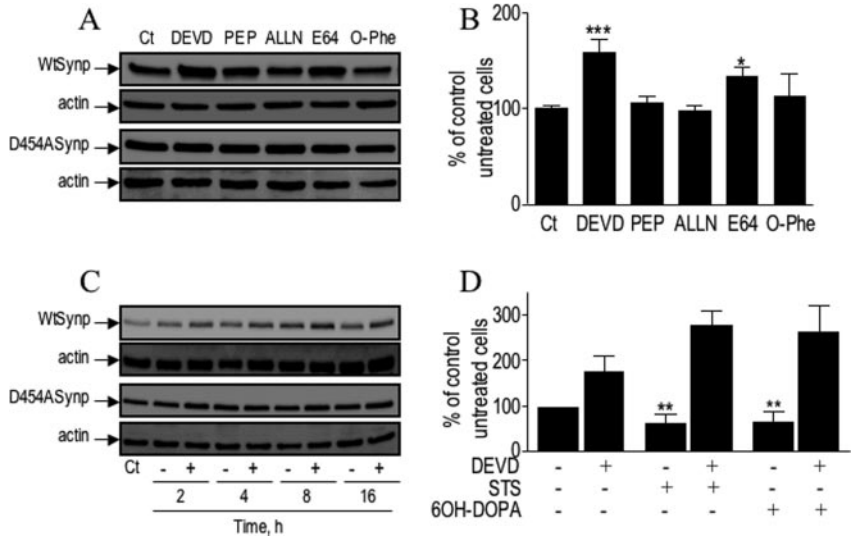

FIGURE 2. **Pharmacological analysis of wild-type and D454A-synphilin-1 degradation in HEK293 stable transfectants.** Wild-type synphilin-1 (*WtSynp*) and mutated D454A-synphilin-1 (*D454ASynp*)-expressing cells were incubated for 16 h (*A*) in the absence (*Ct*) or in the presence of the protease inhibitor Ac-DEVD-CHO (DEVD; 100 μM), pepstatin (PEP; 10 μM), ALLN (100 μM), E64 (100 μM), or *o*-phenanthroline (*o*-Phe; 100 μM) or for various time periods (*C*) with 100 μM Ac-DEVD-CHO, and then synphilin-1-like immunoreactivity was analyzed by Western blot with anti-V5 antibody as described under "Experimental Procedures." Actin immunoreactivity was monitored as a control of protein charge (see "Experimental Procedures"). *B*, quantitative densitometric analysis of wild-type synphilin-1-like immunoreactivity recovered in *A*. *D*, WT-synphilin-1 cells were preincubated for 16 h without (−) or with (+) Ac-DEVD-CHO (DEVD; 100 μM) and then treated with staurosporine (STS; 2 μM) or 6-hydroxydopamine (6OH-DOPA; 0.2 mM), and WT-synphilin-1 expression was analyzed as above. Bars are the means ± S.E. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p <$ 0,005, compared with control untreated cells.

6OH-DOPA (Fig. 4, *B*, *D*, and *H*) stimulate caspase-3 activity in a time- and dose-dependent manner. Interestingly, WT-synphilin-1 expression drastically reduced caspase-3 activity, whereas the D454A mutation drastically reverted this inhibitory control of caspase-3 activity (Fig. 4, *A–D*, *G*, and *H*). Accordingly, STS- and 6OH-DOPA-induced synphilin-1 catabolites were only observed in cells expressing the wild-type protein (Fig. 4*I*). Overall, these data indicate that caspase-resistant D454A-synphilin-1 was unable to modulate cell death in HEK293 cells and, therefore, that the antiapoptotic response elicited by synphilin-1 was controlled by its proteolysis by caspase-3.

In order to further confirm the influence of WT-synphilin-1 on caspase-3 modulation, we analyzed the immunoreactivities of the inactive procaspase-3 in control and STS- or 6OH-DOPA-stimulated conditions. Procaspase-3 is the inactive precursor of caspase-3 that is catalytically activated by caspase-8 and caspase-9 during apoptosis. Thus, a reduction of its immunoreactivity reflects an activation of cell death processes. As expected, STS (Fig. 4*E*) and 6OH-DOPA (Fig. 4*F*) treatment of mock-transfected cells drastically lowers procaspase-3 expression (Fig. 4, *E* and *F*). It should be noted that the extent of proteolytic maturation of procaspase-3 by 6OH-DOPA was more important than the one triggered by STS, in agreement with caspase-3 activity measurements (see Fig. 4, *A–D*, *G*, and *H*). WT-synphilin-1 elicited a reduction of procaspase-3 cleavage in stimulated conditions (Fig. 4, *E* and *F*), whereas D454A-synphilin-1-expressing cells still displayed procaspase-3 reduction (not shown). It should be noted that WT-synphilin-1 reverted procaspase-3 immunoreactivity to nearly control levels (Fig. 4, *E* and *F*). This suggests that the bulk of WT-synphilin-1-induced effects observed on "caspase-like" activities as well as its protection by the Ac-DEVD-CHO indeed reflects a functional link between WT-synphilin-1 and genuine caspase-3 rather than another caspase-like activity.

PARP is an enzyme implicated in the reparation of DNA that is proteolytically inactivated by caspase-3 during apoptosis. Thus, an augmentation of its 89-kDa cleavage product or a lowering of the precursor *versus* product ratio reflects an increase of caspase-3 activity and subsequent caspase-3-dependent apoptotic process. As expected, STS or 6OH-DOPA treatment of mock-transfected cells drastically augments the recovery of PARP product with concomitant virtual abolishment of PARP precursor immunoreactivity (Fig. 4, *E* and *F*). In both STS- and 6OH-DOPA-stimulated conditions, WT-synphilin-1 expression enhances PARP precursor immunoreactivity (Fig. 4, *E* and *F*), thereby leading to an augmentation of precursor *versus* product ratio. Altogether, our data demonstrate by both enzymatic and immunological approaches that WT-synphilin-1 triggers and antiapoptotic response by controlling caspase-3 activity and that this phenotype is fully reverted by site-directed mutagenesis of the synphilin-1 caspase-3 cleavage consensus site.

In order to further delineate the cellular intermediates involved in the WT-synphilin-1 antiapoptotic phenotype, we examined the influence of WT-synphilin-1 on the p53-dependent pathway. Fig. 5 shows that WT-synphilin-1-expressing HEK293 cells display drastically reduced p53 transcriptional activity (Fig. 5*A*) and nuclear expression (Fig. 5*B*). Furthermore, WT-synphilin-1 lowers the transactivation of the p53 promoter (Fig. 5*C*), in very good agreement with the reduced p53 mRNA levels established by real time PCR (Fig. 5*D*). Of most interest is

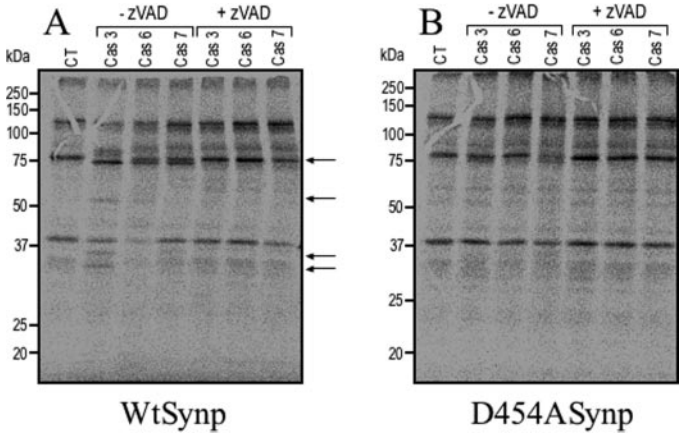

FIGURE 3. **Wild-type but not mutated synphilin-1 is cleaved by recombinant caspase-3 *in vitro*.** WT-synphilin-1 (*A*) or D454A-synphilin-1 (*B*) were transcribed and translated *in vitro* with [^{35}S]methionine and incubated for 8 h at 37 °C with purified recombinant caspase-3, -6, and -7 (25 ng; *Cas 3*, *6*, *7*) in the absence or presence of benzyloxycarbonyl-VAD (10 μM). The reaction mixes were analyzed by SDS-PAGE, and the reaction products (indicated by the *arrows*) were revealed by autoradiography as described under "Experimental Procedures."

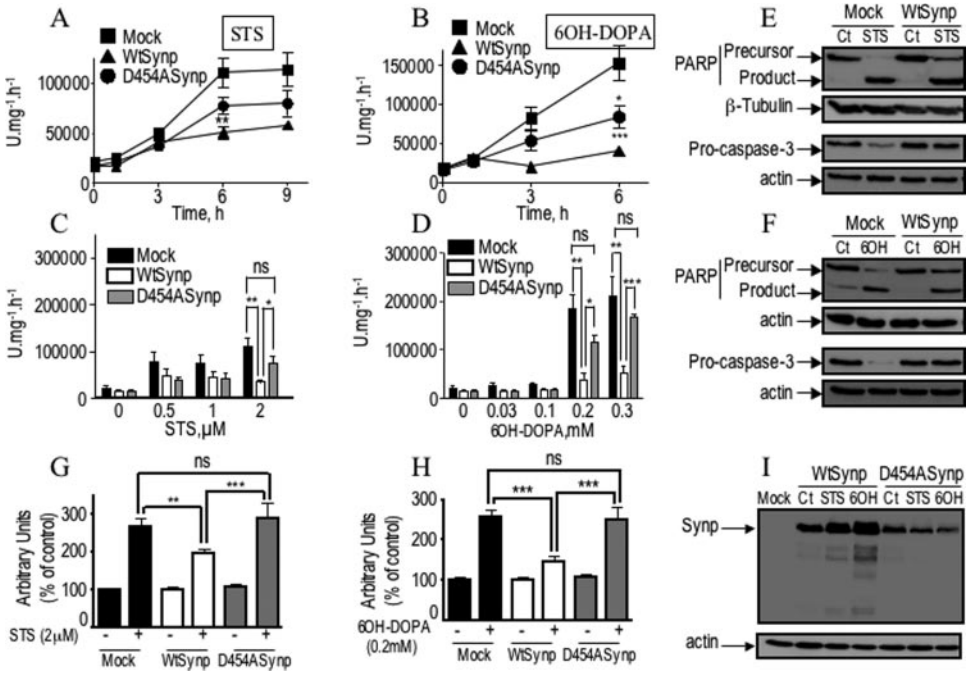

FIGURE 4. **WT-synphilin-1 but not D454A-synphilin-1 reduces staurosporine- and 6OH-DOPA-induced caspase-3 activation in HEK293 cells.** Mock-transfected, WT-synphilin-1 (*WtSynp*)- and D454A-synphilin-1 (*D454ASynp*)-stably expressing HEK293 cells (*A–D*) were treated for the indicated times with staurosporine (2 μM; *A*) or 6OH-DOPA (0.2 mM; *B*) or for 6 h with the indicated concentrations of STS (*C*) or 6OH-DOPA (*D*), and then caspase-3 activity was monitored as described under "Experimental Procedures." In *E* and *F*, WT-synphilin-1-expressing cells were treated for 6 h with either STS (2 μM) or 6OH-DOPA (0.2 mM), and then PARP (precursor and product) and procaspase-3 immunoreactivities were monitored as described under "Experimental Procedures." *G* and *H*, HEK293 cells were transiently transfected with WT-synphilin-1 or D454A-synphilin-1 cDNA. Twenty-four hours after transfection, cells were treated for 6 h with STS (2 μM) or 6OH-DOPA (0.2 mM), caspase-3 activity was monitored as above, and then cells were lysed and analyzed for their synphilin-1-like immunoreactivity as described under "Experimental Procedures" (*I*). *Bars*, means ± S.E. of 3–5 (*A* and *B*), 5–9 (*C* and *D*), or 4–6 (*G* and *H*) independent experiments. ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$. *ns*, nonsignificant. One arbitrary unit (*U*) corresponds to the release of 4 nmol of 7-amino-4-methylcoumarin.

our observation that the down-regulation of the p53 pathway was not observed in cells expressing mutated D454A-synphilin-1 (Fig. 5, *A–D*).

WT-synphilin-1 Reduces STS- and 6OH-DOPA-induced Cell Death in TSM1 Neurons and in SH-SY5Y Neuroblastoma Cells—In order to rule out a problem of cell specificity, we have analyzed the ability of synphilin-1 to modulate cell death in TSM1 neurons and in SH-SY5Y, a cell model particularly relevant to study Parkinson disease (34–37). We have established TSM1 stable transfectants overexpressing WT-synphilin-1 (Fig. 6*A*). As shown in Fig. 6*A*, the immunoreactivity of synphilin-1 is drastically augmented after treatment with Ac-DEVD-CHO, confirming the susceptibility of synphilin-1 to cleavage by caspases in a neuronal cell line. Fig. 6*B* shows that WT-synphilin-1 significantly reduced Ac-DEVD-CHO-sensitive caspase-3 activity in basal conditions. This phenotype was further exacerbated in both STS-stimulated (Fig. 6*B*, *left*) and 6OH-DOPA-stimulated (Fig. 6*B*, *right*) conditions. Fig. 6*C* illustrates the immunological profile of PARP cleavage in mock-transfected and WT-synphilin-1-expressing TSM1 neurons. As expected, STS and 6OH-DOPA treatment of mock-transfected cells led to decreased PARP precursor expression and concomitant detection of a related product, the formation of which was fully prevented by Ac-DEVD-CHO, confirming the implication of caspase-3 on the processing of PARP precursor (Fig. 6*C*). Overexpression of WT-synphilin-1 blocks 70–100% of PARP product formation in STS- and 6OH-DOPA-stimulated conditions, respectively (Fig. 6*C*). Comparative transient transfection analyses (Fig. 6*E*) show that, unlike WT-synphilin-1, D454A-synphilin-1 did not protect TSM1 neurons from STS- and 6OH-DOPA-induced caspase-3 activation (Fig. 6*D*). The latter data were fully confirmed in SH-SY5Y (Fig. 7). Thus, transient transfection of WT-synphilin-1 but not D454A-synphilin-1 coding cDNA (Fig. 7*B*) lowered SH-SY5Y responsiveness to STS (Fig. 7*A*, *left*) and 6OH-DOPA (Fig. 7*A*, *right*). Altogether, these data confirm the susceptibility of WT-synphilin-1 to caspase-3 proteolysis and the ability of this protein, but not its caspase-resistant mutated counterpart, to down-regulate STS- and 6OH-DOPA-stimulated caspase-3 activation in TSM1 neurons and SH-SY5Y neuroblastoma cells.

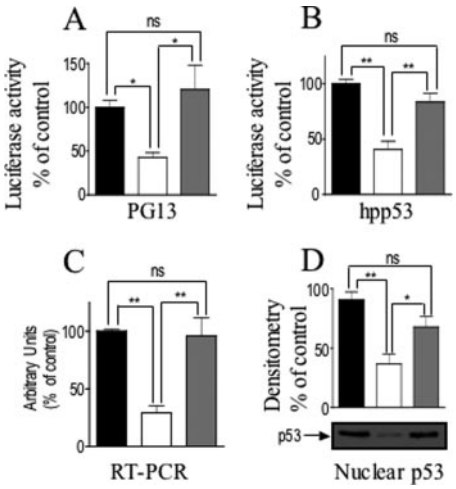

FIGURE 5. **WT-synphilin-1 but not D454A-synphilin-1 down-regulates the p53 pathway in HEK293 cells.** Stably transfected HEK293 cells expressing empty pcDNA3 (*black bars*), WT-synphilin-1 (*white bars*), or D454A-synphilin-1 (*gray bars*) were monitored for their p53 transcriptional activity (*PG13*) (*A*), human p53 promoter (*hpp53*) transactivation (*B*), mRNA levels (real time PCR analysis (*RT-PCR*)) (*C*), and nuclear p53 expression (*D*), as described under "Experimental Procedures." *Bars*, means ± S.E. of 4–9 independent experiments. *, $p < 0.05$; **, $p < 0.01$.

The Caspase-3-derived C-terminal Fragment of Synphilin-1 Lowers HEK293 and TSM1 Responsiveness to STS- and 6OH-DOPA-induced Caspase-3 Activation—The fact that WT-synphilin-1 undergoes caspase-3-mediated proteolysis together with the observation that the mutation that renders WT-synphilin-1 resistant to this cleavage also abolished its antiapoptotic phenotype strongly suggested that the C-terminal fragment of WT-synphilin-1 (synphilin-1-CTF) generated by caspase-3 could indeed be responsible for the WT-synphilin-1-associated protective phenotype. In order to directly examine this possibility, we have designed the V5-tagged synphilin-1-CTF (Fig. 8), and we have assessed its influence after transient transfection in HEK293 cells and TSM1 neurons. Synphilin-1-CTF lowers the STS-induced (Fig. 8, *A* and *C*) and

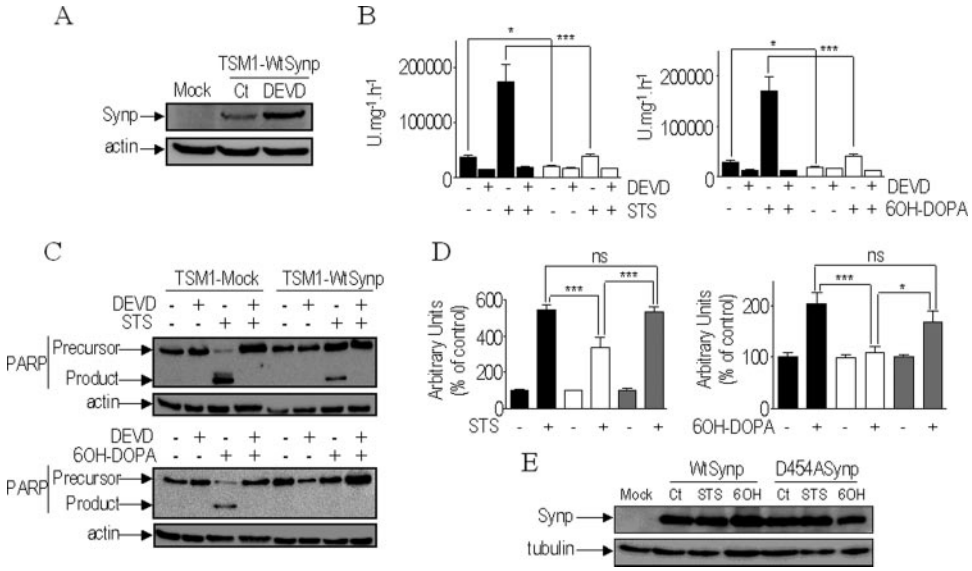

FIGURE 6. **Synphilin-1 but not mutated D454A-synphilin-1 undergoes caspase cleavage and lowers caspase-3 activation in TSM1 neuronal cell line.** *A*, mock- or WT-synphilin-1 (*WtSynp*)-stably transfected TSM1 cells were incubated overnight without (*Ct*) or with Ac-DEVD-CHO (*DEVD*; 100 μM), and then synphilin-1-like immunoreactivity was analyzed by Western blot as described under "Experimental Procedures." *B* and *C*, mock-transfected (*black bars* in *B*) or WT-synphilin-1 (*white bars* in *B*) TSM1 cells were preincubated for 16 h without (−) or with (+) Ac-DEVD-CHO (*DEVD*) and subsequently treated with STS (2 h, 1 μM) or 6-hydroxydopamine (*6OH-DOPA*; 8 h, 0.2 mM), and then caspase-3 activity (*B*) and PARP (precursor and product)- and actin-like immunoreactivities (*C*) were monitored as described under "Experimental Procedures." *D*, TSM1 cells were transiently transfected with empty pcDNA$_3$ (*black bars*), WT-synphilin-1 (*white bars*), or D454A-synphilin-1 (*gray bars*) cDNA. Twenty-four hours after transfection, cells were treated with STS (2 h, 1 μM) or 6OH-DOPA (8 h, 0.2 mM), and then caspase-3 activity was monitored as above. Cells were lysed and analyzed for their synphilin-1- and tubulin-like immunoreactivities (*E*). The *bars* are the means ± S.E. of 8–13 (*B*) or 4–5 (*D*) independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

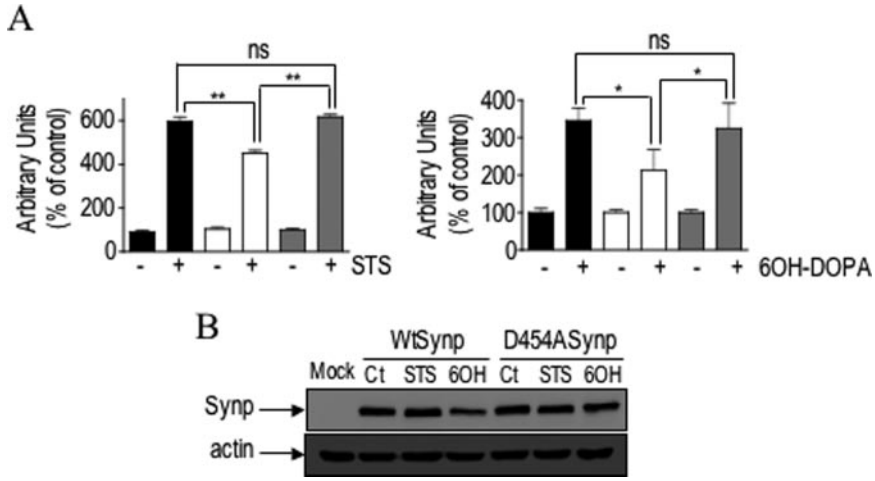

FIGURE 7. **Synphilin-1 but not mutated D454A-synphilin-1 lowers staurosporine- and 6OH-DOPA-induced caspase-3 activation in the SH-SY5Y neuroblastoma cell line.** SH-SY5Y cells were transiently transfected with empty $pcDNA_3$ (*black bars*), WT-synphilin-1 (*white bars*), or D454A-synphilin-1 (*gray bars*) cDNA. Twenty-four hours after transfection, cells were treated with STS (2 h; 1 μM; *left*) or 6OH-DOPA (8 h; 0.2 mM; *right*), and then caspase-3 activity was monitored as described under "Experimental Procedures" (*A*). Cells were lysed and analyzed for their synphilin-1-like and actin immunoreactivities (*B*). *Bars*, means ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.001$.

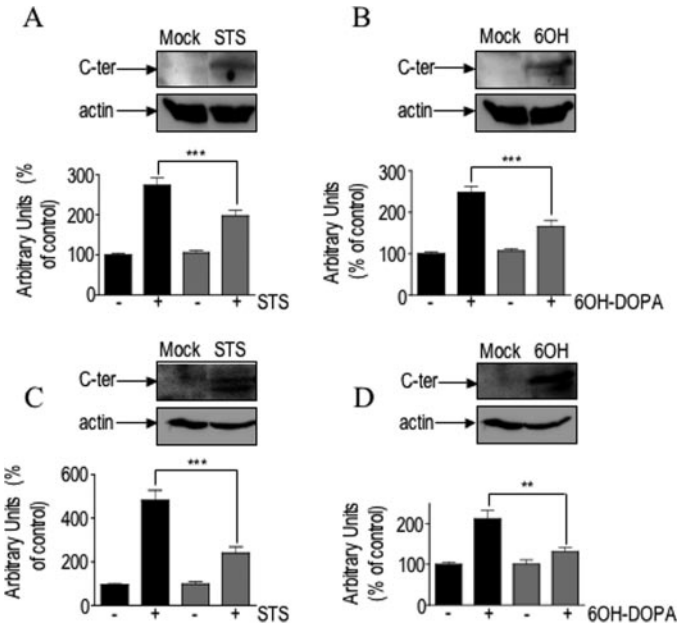

FIGURE 8. **The caspase-3-derived C-terminal fragment of synphilin-1 lowers staurosporine- and 6OH-DOPA-induced caspase-3 activation in HEK293 and TSM1 cell lines.** HEK293 cells (*A* and *B*) and TSM1 neurons (*C* and *D*) were transiently transfected with empty $pcDNA_3$ (*black bars*) or WT-synphilin-1 C-terminal fragment (*gray bars*) cDNA. Twenty-four hours after transfection, cells were treated with STS (2 μM for 6 h (*A*) and 1 μM for 2 h (*C*)) or 6OH-DOPA (*6OH*; 0.2 mM for 6 h (*B*) and 0.2 mM for 8 h (*D*)), and then caspase-3 activity was monitored as described under "Experimental Procedures." Cells were lysed and analyzed for their synphilin-1-like and actin immunoreactivities (*A–D*, *insets*). *Bars*, means ± S.E. of 4–8 independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

6OH-DOPA-induced (Fig. 8, *B* and *D*) caspase-3 activation in both HEK293 cells (Fig. 8, *A* and *B*) and TSM1 neurons (Fig. 8, *C* and *D*).

DISCUSSION

PD-affected brains exhibit selective loss of substantia nigra pars compacta neurons and are invaded at late stages by cytoplasmic inclusions called Lewy bodies (LB) (38–40). Dopaminergic neuron cell death is apparently linked to exacerbated oxidative stress and p53-dependent apoptosis (41–44) that could be the consequence of the accumulation and aggregation of misfolded proteins. Thus, it has been demonstrated that aggregated proteins display inherent toxicity (45) and harbor the ability to inhibit the proteasome (46). In this context, when the cellular capacity of refolding, recovery, and degradation are saturated, misfolded proteins accumulate, aggregate (47), and ultimately kill the cells.

LB reflect such an accumulation process in PD. These structures are mainly composed of ubiquitin, a number of elements of the proteosomal machinery and aggregated proteins among which α-synuclein is the main component (48). α-Synuclein, one of the key proteins implicated in familial PD (10, 49, 50), has a high propensity to aggregate *in vitro* and *in vivo*, and several studies showed that α-synuclein aggregation can be exacerbated by pathogenic mutations and by different factors, including the dopaminergic derivative prooxidant toxin 6OH-DOPA (for reviews, see Refs. 51–53). Interestingly, α-synuclein aggregation impairs its function. Thus, the A53T familial-associated PD mutation and 6OH-DOPA both trigger α-synuclein aggregation and abolish its antiapoptotic function (13).

α-Synuclein displays remarkable chaperone properties (53), and recently, synphilin-1 has been characterized as one of its binding partners (1, 19). Interestingly, synphilin-1 accumulates in LB (19), and the co-overexpression of α-synuclein and synphilin-1 favors the formation of eosinophil cytoplasmic inclusions that resemble LB (1, 21). Therefore, the possible implication of synphilin-1 in the formation of the LB and its possible functional link with α-synuclein led us to study the role of synphilin-1 in cell death.

We have established that wild-type synphilin-1 has a protective phenotype in human HEK293 cells, TSM1 neurons, and SH-SY5Y neuroblastoma cells. Thus, synphilin-1 reduces STS- and 6OH-DOPA-induced caspase-3 activation and PARP cleavage. In agreement with its protective function, WT-synphilin-1 also drastically down-regulated the proapoptotic p53 pathway. Interestingly, synphilin-1 function appears regulated by its proteolysis. Thus, we show that cellular synphilin-1 degradation is enhanced by the proapoptotic effectors STS and 6OH-DOPA and reduced by caspase-3 inhibitor. In agreement, we found that synphilin-1 is cleaved preferentially by caspase-3 *in vitro*. Interestingly, D454A-synphilin-1, a mutant in which a consensus cleavage site for caspase-3 had been abolished, fully resisted proteolysis by recombinant caspase-3.

What is the molecular influence of caspase-3 cleavage on synphilin-1 function? At least two theoretical hypotheses could stand. First, synphilin-1 holoprotein itself would be responsible for the protective phenotype, and caspase-3 cleavage could be seen as an inactivating process. Second, synphilin-1-associated antiapoptotic phenotype would be associated with one of its caspase-3-derived proteolytic products. Our data strongly argue in favor of the latter view. Thus, synphilin-1-induced antiapoptotic phenotype is drastically reduced when synphilin-1 is rendered resistant to caspase-3 proteolysis by mutagenesis of a caspase-3 cleavage site consensus sequence. This observation strongly suggested a role of caspase-3 in the generation of a synphilin-1-derived product with

antiapoptotic properties. Indeed, we have shown that the caspase-3-derived C-terminal fragment of synphilin-1 lowered staurosporine- and 6OH-DOPA-induced caspase-3 activation. In this context, one could envision that cellular stress or environmental factors trigger caspase-3 activation and associated cell death but also provide a means to down-regulate apoptosis by concomitantly increasing the production of caspase-3-derived synphilin-1 proteolytic fragment. It should be noted that this type of regulation has already been documented for other proteins. Thus, presenilins (54, 55) and β-amyloid precursor protein (56–60) undergo caspase-derived cleavages, generating proteolytic fragments controlling cell death. More related to PD, parkin, another binding partner of synphilin-1 (18, 61) displaying an antiapoptotic phenotype (62, 63), is also cleaved by caspases, but unlike for synphilin-1, this endoproteolysis leads to a loss of function of this protein (64).

It is worth noting that although both α-synuclein and synphilin-1 protect human cells and neurons from STS stimulation (12, 13), only synphilin-1 keeps its protective function in the presence of 6OH-DOPA (this work). This phenotype is reminiscent of the one associated with β-synuclein, the homologue of α-synuclein. Thus, both synphilin-1 and β-synuclein remain protective toward 6OH-DOPA (55) and lower the p53 pathway. Furthermore, β-synuclein restores the protective activity of α-synuclein, even in the presence of 6OH-DOPA (55). Whether synphilin-1 restores the antiapoptotic potential of α-synuclein in the presence of the dopaminergic derivative remains to be established. However, it should be noted that α-synuclein and synphilin-1 co-localize in LB at the late stages of the pathology and that aggresomes formed by α-synuclein and synphilin-1 are cytoprotective (65). These observations together with the present demonstration of a protective function of synphilin-1 argue in favor of a caspase-3-regulated protective role of synphilin-1 and for a functional cross-talk between α-synuclein and synphilin-1 within LB.

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B.II) La fonction protectrice de DJ-1 est abolie par des mutations pathogènes (Article 2)

Article 2:"Loss of function of DJ-1 triggered by Parkinson's disease-associated mutation is due to proteolytic resistance to caspase-6." En révision

Actuellement la fonction de DJ-1 est encore mal connue, nous avons donc essayé de mieux comprendre son rôle dans les processus apoptotiques et, en particulier, l'influence de certaines mutations pathogènes sur cette fonction.

Les cellules surexprimant DJ-1 sauvage sont moins sensibles à l'apoptose

Pour cette étude nous avons établi des lignées cellulaires surexprimant stablement DJ-1 sauvage, DJ-1 mutée contenant soit la mutation D149A qui se situe sur un site de clivage putatif des caspases, soit la mutation L166P dans des cellules neuronales murines (TSM1) ou des cellules dopaminergiques humaines (SH-SY5Y). Les cellules TMS1 et SH-SY5Y surexprimant stablement ou transitoirement DJ-1 sauvage sont plus résistantes à l'apoptose induite par la 6-OHDA. En effet, dans ces cellules la surexpression de DJ-1 sauvage induit une diminution de l'activation des caspases effectrices ainsi qu'une protection du précurseur actif de PARP (une enzyme de réparation de l'ADN), de façon intéressante, les mutations D149A et L166P abolissent cette fonction protectrice (Figure 1).

DJ-1 sauvage est capable de réguler la voie p53.

Au cours de ce travail, nous avons déterminé que la fonction protectrice de DJ-1 passe par la régulation de la voie dépendante de p53. En effet, DJ-1 est capable de réguler négativement aussi bien l'expression que l'activité de p53. Nous avons voulu savoir comment et à quel niveau DJ-1 régule p53, nous avons donc vérifié l'effet de DJ-1 sur la transactivation du promoteur de p53 ainsi que son influence sur le taux d'ARN de p53. Tout comme pour l'expression et l'activité de p53, nous avons observé une diminution de la transactivation de son promoteur et de son niveau

d'ARN messager (Figure 2A). En outre, DJ-1 semble séquestrer p53 dans le cytoplasme diminuant ainsi son expression dans le noyau (Figure 2C) et donc son activité mais également activer la voie Akt qui conduit à l'augmentation de la dégradation de p53 (Figure 3A). La régulation transcriptionnelle et post-transcriptionnelle sur p53 qu'exerce DJ-1 sauvage n'est pas visible avec les deux mutants. Il est également apparu que, dans des cellules dépourvues de p53, DJ-1 est incapable de protéger les cellules d'un stimulus apoptotique, sa fonction passe non seulement par la régulation de la voie p53 mais également est dépendante de cette voie (Figure 2B).

L'invalidation de DJ-1 dans des cellules ou dans des souris conduit à l'augmentation de l'apoptose (augmentation de l'activité de la caspase-3) corrélée à l'augmentation de l'expression et de l'activité de p53, ceci est inversé par la complémentation de ces cellules avec l'ADNc de DJ-1 sauvage (Figure 4).

L'activité protectrice de DJ-1 est portée par son fragment C-terminal.

Dans la littérature, la mutation L166P est décrite comme déstabilisant la structure tridimensionnelle de DJ-1 et induisant une instabilité conduisant à sa dégradation rapide (Olzmann et al., 2004). Par contre, l'effet de la mutation D149A sur la fonction de DJ-1 est mal connu. Nous avons observé que cette mutation à l'inverse de la mutation L166P conduit plutôt à l'agrégation DJ-1. Une étude de la séquence de DJ-1 nous a permis de mettre en évidence un site putatif de clivage par des caspases. Des études *in vitro* ont mis en évidence une coupure de DJ-1 par la caspase-6, le site de cette coupure correspondant à la mutation D149A (Figure 5). Nous avons donc produit le fragment issu du clivage de DJ-1 par la caspase-6. Ce fragment C-terminal possède la même fonction protectrice que DJ-1 sauvage, ce fragment diminue l'activité de la caspase-3 induite par une stimulation à la 6-OHDA, en diminuant la voie dépendante de p53 (Figure 6).

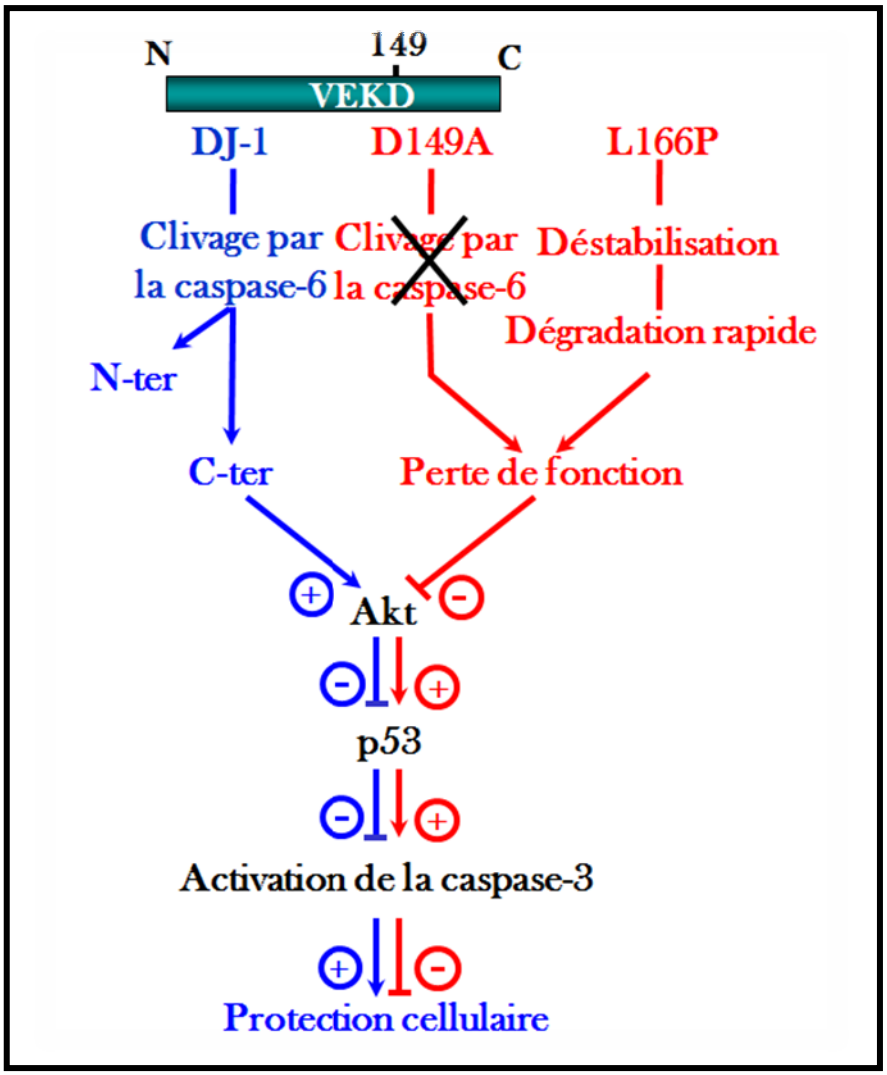

Figure 39 : Schéma récapitulant les mécanismes impliqués dans la fonction protectrice de DJ-1

De façon intéressante, le taux de caspase-6 est augmenté dans les cerveaux de patients atteints par des formes sporadiques de la maladie, en corrélation avec une légère diminution du niveau de DJ-1 (Figure 7).

Conclusion/Discussion

DJ-1 protège donc les cellules de l'apoptose en régulant la voie dépendante de p53 à des niveaux transcriptionnel et post-transcriptionnel (Figure 39). Cette fonction protectrice qui passe par l'activation de la voie Akt est également dépendante de la présence de p53. De plus les mutations pathogènes L166P et D149A aboutissent toutes les deux à une perte de fonction de DJ-1 mais par des mécanismes différents. En effet, la mutation déstabilise la structure de DJ-1 et conduit à sa dégradation rapide il n'y a donc pas production du fragment C-terminal, alors que la mutation D149A inhibe le clivage de DJ-1 par la caspase-6 et empêche donc la formation du fragment C-terminal protecteur de DJ-1 provocant également l'agrégation de DJ-1 (Figure 8). Des études récentes ont confirmé l'implication de p53 dans la fonction de DJ-1 (Bretaud et al., 2007; Shinbo et al., 2005).

Article 2

Giaime E., Sunyach C., Druon C., Robert G., Grosso S, Auberger P., Goldberg M.S., Shen J., Heutink P., Pouysségur J., Pagès G., Checler F., and Alves da Costa C.

"Loss of function of DJ-1 triggered by Parkinson's disease-associated mutation is due to proteolytic resistance to caspase-6."

En révision

Loss of function of DJ-1 triggered by Parkinson's disease-associated mutation is due to proteolytic resistance to caspase-6

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Abstract

DJ-1 was recently identified as a gene product responsible for a subset of familial Parkinson's disease (PD). The mechanisms by which mutations in DJ-1 alter its function and account for PD-related pathology remained largely unknown. We show that DJ-1 is processed by caspase-6 and that the caspase-6-derived C-terminal fragment of DJ-1 fully accounts for associated p53-dependent cell death. In lines with the above data, we demonstrate that a recently described mutation (D149A) associated with early onset PD renders DJ-1 resistant to caspase-6 proteolysis and abolishes its protective phenotype. Unlike the D149A mutation, the L166P mutation that prevents DJ-1 dimerization does not impair its proteolysis by caspase-6 although it also abolishes DJ-1 anti-apoptotic function. Therefore, we demonstrate here that DJ-1 loss of function could be due to impaired caspase-6 proteolysis and we document the fact that various DJ-1 mutations could lead to PD pathology through distinct molecular mechanisms.

Introduction

Parkinson's disease (PD) is a movement disorder, the incidence of which increases sharply with age. It is characterized by a massive loss of dopaminergic neurons of the substantia nigra pars compacta and the presence of intra-cytoplasmic inclusions named Lewy bodies (LB). Most of PD cases are of sporadic origin but about five percent of them are of genetic origin and are either associated to an autosomal dominant or recessive mode of transmission. The latter forms of the disease are usually associated to an early onset (<50 years-old) and are linked to mutations in the genes of parkin, PINK-1 and DJ-1 (Gasser, 2005).

DJ-1 is implicated in approximately 1-2% of recessive forms of PD (Abou-Sleiman et al., 2003; Hedrich et al., 2004) and is a ubiquitous highly conserved protein that is normally expressed in the brain as a homodimeric complex (Bandopadhyay et al., 2004). To date, only two DJ-1 mutations corresponding to a deletion of exons 1-5 and a point mutation that converts the leucine residue in position 166 into a proline (referred to as L166P-DJ-1 hereafter), have been identified in a Dutch and an Italian family, respectively. That these DJ-1 mutations triggered a drastic decrease of DJ-1 levels suggested that these familial cases were likely due to a loss of function of DJ-1. In apparent contradiction with these conclusions, recently, a heterozygous DJ-1 mutation (D149A) was reported to lead to early-onset PD while DJ-1 appeared catabolically stabilized (Abou-Sleiman et al., 2003; Takahashi-Niki et al., 2004).

Little is known about the physiological function of DJ-1 and the mechanisms by which DJ-1 mutations lead to PD although invalidation of DJ-1 clearly established dopaminergic deficits and hypokinesia (Goldberg et al., 2005). A few functional studies indicated that, as a member of the ThiJ/PfpI family, DJ-1 could act as a molecular chaperone (Lee et al., 2003). It has also been suggested that DJ-1 may possess RNA binding properties (Hod et al., 1999) and

may lead to transcriptional activation through the interaction with PIASx (Araki et al., 2001) that is an ubiquitin-ligase involved in the process of sumoylation of several proteins (Palvimo, 2007). It should be noted that DJ-1 itself is sumoylated, suggesting a role of this protein in cell signaling (Shinbo et al., 2005). Several lines of evidence also indicate that DJ-1 may act as an oxidative stress sensor. Thus, it has been demonstrated that the cysteine 106 of DJ-1 is essential for its acidic PI shift in oxidative stress conditions (Canet-Aviles et al., 2004; Kinumi et al., 2004). Of most interest, the antioxidant properties of DJ-1 are associated to its ability to trigger neuroprotection (Taira et al., 2004).

The mechanisms by which DJ-1 elicits neuroprotection are far from being elucidated. Here we show that the over-expression of DJ-1 in neuronal and dopaminergic cells elicits a p53-dependent protective response against various PD and non PD-associated stimuli. Thus, cells over-expressing wild-type DJ-1 display decreased p53 expression, promoter transactivation and mRNA levels by an Akt-dependent signaling while conversely, DJ-1 depletion triggers an up-regulation of the p53 pathway in both cellular and knockout animal models. Functional comparison studies between L166P-DJ-1 and D149A-DJ-1 indicate that both mutations abolish DJ-1-associated control of p53. However, L116P-DJ-1 and D149A-DJ-1 display distinct susceptibility to caspase-6. Thus, unlike L166P, the D149A mutation fully blocks DJ-1 cleavage by recombinant caspase-6. This proteolytic resistance fully explains the loss of function of DJ-1 since we demonstrate that the C-terminal fragment of DJ-1 derived from its cleavage by caspase-6 totally accounts for the DJ-1-mediated protective function. Interestingly, we show that the levels of DJ-1 and caspase-6 are inversely correlated in human brain samples derived from sporadic PD patients, suggesting a role of caspase-6 in the physiological control of DJ-1 brain levels and its loss of function in the pathology. Overall, this is the first demonstration of the selective implication of caspase-6 in the

proteolysis of DJ-1 and that the blockade of this process is likely responsible for a subset of autosomal recessive early onset PD cases.

Experimental procedures

Antibodies and Materials

The anti-V5 mouse monoclonal antibody and lipofectamine reagent were purchased from Invitrogen (Invitrogen Corporation, CA). Anti-DJ-1 rabbit polyclonal antibodies (ab18257 and ab37180 used for western blot and co-immunoprecipitation analysis, respectively) and anti-caspase-6 monoclonal antibodies (ab17866) were purchased from Abcam (Abcam, Paris, France). Anti-human total p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin and anti-actin monoclonal antibodies, staurosporine (STS), 6-hydroxydopamine (6OHDA), cycloheximide, Ac-DEVD-aldehyde, Ac-DEVD-7-amino-4-methylcoumarin, ALLN (N-acetyl-L-leucyl-L-norleucinal), AEBSF [4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride], E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], lactacystin, pepstatin, *o*-phenanthroline and calpain inhibitor I were purchased from Sigma (St. Quentin-Fallavier, France). LY294002 was purchased from Cayman Chemicals (Ann Arbor, Michigan). The IκB kinase inhibitor BMS-345541 was purchased form Calbiochem (Merck Chemicals Limited, Nottingham, England). Anti-human PARP antibodies were purchased from Euromedex (Souffelweyersheim, France). The anti-active caspase-3 rabbit polyclonal antibody was purchased, R&D Systems (R&D Systems, Minneapolis, USA). S17092 (Barelli et al., 1999) was kindly provided by Philippe Morain (Servier, Paris). Benzylocarbonyl-Ile-Leu-(OBut)-Ala-leucinal (ZIE) and benzyloxycarbonyl-leucinal (ZL) were kindly provided by Dr S. Wilk (Mount Sinaï , New York, USA).

Site Directed Mutagenesis

D149A-DJ-1 and D149A/L166P-DJ-1 were obtained by site-directed mutagenesis from wild-type DJ-1 and mutated L166P-DJ-1 V5-tagged cDNA by means of QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The two primers containing the

D149A pathogenic mutation 5'-CGTGTGGAAAAAGCAGGCCTGATTCTTACAAGC-3' and 5'-GCTTGTAAGAATCAGGCCTGCTTTTTCCACACG-3' (Eurogentec, Angers, France) were designed according to the manufacturer's recommendations. The D60A-DJ-1, the D60A/D149A-DJ-1 and the D60A/D149A/L166P-DJ-1 were obtained by oligonucleotide-directed mutagenesis from wild-type DJ-1, mutated D149A-DJ-1, and mutated D149A/L166P-DJ-1 V5-tagged cDNAs by means of QuikChange^{TM} site-directed mutagenesis kit (Stratagene). The two primers containing the D60A mutation were as follows: 5'-GCCTTGAAGCAGCAAAAAAAGAGGG-3' and 5'-CCCTCTTTTTTTGCTGCTTCAA GGC-3' (Eurogentec) were designed according to the manufacturer's conditions. The cDNA encoding the V5-tagged caspase-6-derived N-terminal fragment of DJ-1 was engineered by introducing a Xho restriction site in position 150, i.e after the aspartyl residue in position 149 involved in a putative consensus cleavage site of caspase-6 (oligonucleotide 5' CTAGACTCGAGTCTTTCACACG-3'), to allow the subcloning of the construction in pcDNA3.1/V5/His-TOPO. The C-terminal fragment of DJ-1 was obtained by introducing an ATG codon in position 149 (oligonucleotide 5'-TAATTAAGCTTATG GGCCTGATTCTTACAAGCCGG-3'). An additional HindIII restriction site was also added, adjacent to the ATG codon, for further subcloning of the construction in pcDNA3.1/V5/His-TOPO.

Cell Systems and Transfections

Telencephalon Specific Mouse 1 (TSM1), Human Embryonic Kidney 293 cells (HEK293), SH-SY5Y human neuroblastoma cells, and Mouse Embryonic Fibroblasts (MEF) were cultured in Dulbecco's media supplemented with 10% fetal bovine serum. Stable transfectants expressing empty vector, wild-type, and mutated DJ-1 in TSM1 cells were obtained after transfection with 2µg of each cDNA (all in pcDNA3) by means of

Lipofectamine according to the manufacturer's conditions. Positive clones were screened for their DJ-1-like immunoreactivity and mRNA levels as described below. Transient transfections of SH-SY5Y, DJ-1 knockout MEF cells (Goldberg et al., 2005), $p19^{Arf}$-deficient and $p19^{Arf}$/p53 double knockout MEF cells (Kamijo et al., 1997) and ERK-1 knockout MEF cells (Pages et al., 1999) were carried by means of either lipofectamine reagent or Nucleofector™ kit according to the manufacturer's instructions (Amaxa Biosystems, Koeln, Germany) as previously described (Sunyach et al., 2007).

Primary cultures of neurons

Embryonic cortical neurons were prepared as previously described (Vincent et al., 1996). Briefly, cells from cerebral hemispheres of C57B mouse embryos (E14 stage) were dissociated in Ham's F12 (Fisher Bioblock Scientific, Illkirch, France), supplemented with 0.6% glucose and 10% foetal calf serum. A total of $5x10^6$ cells were transfected with 3µg of each cDNA (all in pcDNA3) by means Mouse Neuron Nucleofector™ kit according to the manufacturer's instructions (Amaxa Biosystems, Koeln, Germany) and were seeded in 12-well plates pre-coated with 10µg/ml polylysine (Sigma).

Western Blot Analysis

Cells were homogenized in lysis buffer 10mM Tris-HCl pH7.5, containing 150mM NaCl , 0.5%, Triton X-100, 0.5% deoxycholate and 5mM EDTA and resolved on 12% SDS-polyacrylamide gel electrophoresis (PAGE) for the analysis of wild-type and mutated tagged-DJ-1, endogenous DJ-1, total p53 and active caspase-3 or on 8% SDS-PAGE for the detection of poly-ADP-ribose polymerase (PARP). For analysis of the immunoreactivities of the N- and C-terminal fragments of DJ-1, proteins were separated on 16.5% Tris/tricine gels. After electrophoresis, proteins were wet transferred to nitrocellulose membranes and incubated

overnight with the adequate primary antibodies (see above). Protein immunoreactivities were revealed with either an anti-rabbit peroxidase or an anti-mouse peroxidase (Jackson Immunoresearch, Cambridgeshire, UK) by the electrochemiluminescence method as previously reported (Alves da Costa et al., 2006). Chemiluminescence was recorded using a luminescence image analyzer LAS-3000 (Raytest, Courbevoie, France), and quantification of captured images was performed using the Aida Image Analyzer software (Raytest).

I*n Vitro* transcription/translation of wild-type and mutated DJ-1 and cleavage by recombinant caspase-3, -6, and -7 in a cell-free System

Wild-type and mutated DJ-1 were transcribed and translated by means of the Promega TNT coupled reticulocyte lysate system (Shang et al.) in the presence of radiolabeled methionine (ICN) as described (Giaime et al., 2006). Briefly, recombinant caspases-3, -6, or -7 (25ng, Sigma) were incubated for 16 h at 37°C with 2µl of reticulocyte lysates in 50µl of 25mM HEPES (4-(2-hydroxyethil)piperazine-1-ethanesulfonic acid) buffer pH 7.5 containing 0.1% CHAPS ((3-[(3-cholamidopropyl)dimetylammonio]-1-propanesulfonate)) and 5mM dithiothreitol. In a subset of experiments, the caspase inhibitor Acetyl-Aspartyl-Glutamyl-Valine-Aspartyl-aldehyde (Ac-DEVD-CHO) (10µM) was preincubated with caspases prior to the addition of the reticulocyte lysates. Proteins were then electrophoresed on 12% PAGE and autoradiographed using Amersham Biosciences hyperfilms.

Caspase-3 activity measurements

Cells were grown in 6-well plates and incubated without or with staurosporine (STS) or 6-hydroxydopamine (6OHDA) for various time and concentrations. In some cases, cells were pre-treated or not for 30 min with LY294002 (10µM). Caspase-3-like activity was fluorimetrically measured as extensively detailed (Alves da Costa et al., 2000).

Real-time quantitative PCR

Total RNA from cells was extracted using the RNeasy kit following the instructions of the manufacturer (Qiagen, Hilden, Germany). After, DNase I treatment, 2μg of total RNA was reverse-transcribed using oligo (dT) priming and avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR was performed in an ABI PRISM 5700 Sequence Detector System (Applied Biosystems, Foster City, CA), using the SYBR Green detection protocol recommended by the manufacturer. Specific-gene primers were designed by means of the Primer Express software (Applied Biosystems) and are the following: mouse p53-specific primers: forward, 5'-TCCCATCACTTCACTCCTCC-3' and reverse, 5'-AAAAGGCAG CAGAAGGG-3'; V5-specific primers for V5-tagged-DJ-1: forward, 5'-GCGGTTCGAAGG TAAGCCTA-3' and reverse, 5'-GCGGTAGAATCGAGACCGAG-3'; mouse DJ-1-specific primers: forward, 5'-GGAGATGCAAAAACGCAGGG-3' and reverse, 5'-TCCTCCTGG AAGAACCACCA-3'. Relative expressions levels of p53, DJ-1 and V5-tagged-DJ-1 genes were normalized for RNA concentrations with the housekeeping gene mouse γ-actin using the following primers: forward, 5'-CACCATCGGTTGTTAGTTGCC-3', and reverse, 5'-CAG GTGTCGATGCAAACGTT-3'.

Semi quantitative PCR analysis of N-Cas, WTC-Cas and 166C-Cas mRNA

One μg of total RNA obtained as above was used to amplify DJ-1 caspase-derived products by means of Access RT-PCR System kit (Promega) according to manufacturer conditions. The PCR reactions were performed with either V5-specific primers: forward, 5'-GCGGTTCGAAGGTAAGCCTA-3' and reverse, 5'-GCGGTAGAATCGAGACCGAG-3' or GAPDH (control housekeeper gene) forward, 5'-TGGGCTACACTGAGCACCAG-3', and

reverse, 5'-CAG-CGTCAAAGGTGGAGGAG-3'. PCR products were analysed on a 2% agarose gel stained with ethidium bromide.

p53 activity, expression and promoter transactivation

The p53 activity was measured by means of the two p53 reporter genes $p21^{waf-1}$-luciferase and PG13-luciferase constructs (El-Deiry et al., 1992) (provided by Dr. B. Vogelstein, Baltimore, MD) as previously described (Alves da Costa et al., 2006).The transcriptional activation of the human p53 promoter (*hpp53*) (Ginsberg et al., 1990) was measured after transfection of the cDNA coding for the human p53 promoter sequence in frame with luciferase (provided by Dr. M. Oren, Rehovot, Israël) as previously described (Alves da Costa et al., 2003). All activities were measured after co-transfection of the above cDNAs (0.5-1μg) and β-galactosidase (0.25-0.5μg) cDNA, in order to normalize transfection efficiencies (Promega, Madison, WI).

p53 immunoreactivity was analyzed by Western blot using an anti-p53 mouse monoclonal antibody (1:5000 dilution) in nuclear extracts prepared as previously described for cytochrome *c* translocation experiments (Alves da Costa et al., 2006).

Co-immunoprecipitation experiments

Mouse Embryonic Fibroblasts were lysed in 10mM Tris-HCl pH7.5 buffer containing 150mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5mM EDTA and a protease inhibitors cocktail (Sigma, P2714, according to manufacturer's conditions) gently homogenized and centrifuged at 14 000 rpm in order to remove cellular debris. Co-immunoprecipitations of DJ-1 and p53 were performed using ExactaCruz™ IP/Western Blot kit following the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were

resolved on 12% SDS-PAGE and DJ-1 and p53 immunoreactivities were analyzed with the adequate primary (see above) monoclonal or polyclonal antibodies.

Analysis of DJ-1 and p53 expression in mouse brain tissues

DJ-1 and caspase-6 knockout mice have been recently described (Le et al., 2002; Goldberg et al., 2005; Zandy et al., 2005)**.** All brain samples were homogenized with a Potter apparatus in 10mM Tris-HCl pH7.5 buffer complemented with a protease inhibitor cocktail and resolved on 12% SDS-PAGE gels. DJ-1 and p53 immunoreactivities were detected as described above.

Normal and Pathological Human Brain Tissues

All human substantia nigra brain samples were obtained from the GIE Neuro-CEB network (La Pitié-Salpêtrière, Paris, France). These samples include four SLA patients (control non-PD-associated pathology): 1811 (male, 55 years-old), 1821 (female, 68 years-old), 1822 (male, 64 years-old), 1823 (female, 62 years-old), one sample from an aged matched healthy control patient 3659 (male, 61 years-old), and five samples from PD patients: 3605 (male, 64 years-old), 4489 (male, 75 years-old), 4513 (female, 77 years-old), 5193 (male, 75 years-old) and 8460 (male, 66 years-old). The mean post-mortem delay was 21.8 ± 8.5 h. Samples were homogenized with a Potter apparatus in approximately 300µl of lysis buffer 10mM Tris-HCl pH7.5 containing a protease inhibitor cocktail (Sigma). Equal amounts of protein were then resolved on 12% SDS-PAGE gels and incubated overnight with anti-DJ-1, p53 and caspase-6 antibodies.

Statistical Analysis

Statistical analyses were performed with PRISM software (GraphPad Software, San Diego, CA) by using the Newmann-Keuls multiple comparison tests for one-way analysis of variance or the unpaired Student's test for pair wise comparisons.

Results

PD-associated mutations abolish the protective phenotype of DJ-1 in mouse and human neuronal cells.

We have obtained stable transfectants over-expressing wild-type DJ-1 (WTDJ), D149A-DJ-1 (149DJ) and L166P-DJ-1 (166DJ) in TMS1 neurons. The WTDJ and 149DJ clones chosen for experiments express similar DJ-1 proteins (Fig.1A upper) and mRNA levels (Fig. 1A, lower). Although similar mRNA levels were observed for L166P-DJ-1 (Fig.1A lower) reflecting equivalent transfection efficiencies, only longer exposure of SDS-PAGE analysis allowed to visualize the protein, confirming the previously reported catabolic instability of L166P-DJ-1 (Moore et al., 2003). In order to evaluate the ability of DJ-1 and its mutated congeners to control cell death, we have challenged the cells with 6-hydroxydopamine (6OHDA), a natural toxin frequently used to mimic PD in cellular and animal models (Blum et al., 2001). Fig.1B shows that 6OHDA strongly activates caspase-3 in mock-transfected cells. Clearly, WT-DJ-1 drastically reduces 6OHDA-stimulated caspase-3 activation while L166P and D149A mutations abrogate this phenotype. These results were further confirmed by the analysis of the cleavage of PARP, a physiological substrate of caspase-3 that is cleaved and inactivated during apoptosis (Lazebnik et al., 1994). As expected, 6OHDA lowers PARP precursor expression and increases its proteolytic product in mock-transfected cells (Fig.1C). Here again, WT-DJ-1 but not the L166P-DJ-1 and D149A-DJ-1 reduces PARP cleavage confirming that WT-DJ-1 protects TSM1 cells from 6OHDA-

induced caspase-3 activation. This phenotype is not cell-specific. Thus, in human SH-SY5Y neuroblastoma cells, which are considered as one of the most relevant dopaminergic cell model, the over-expression of WT-DJ-1 reduces cellular responsiveness to 6OHDA (Fig.1D) and STS (not shown) while both DJ-1 mutations abolished this phenotype (Fig.1D).

Wild-type but not mutated DJ-1 down-regulates p53 at both transcriptional and post-transcriptional levels.

We have analyzed the potential of WT-DJ-1 and its mutants as modulators of the p53 pathway. By means of a construct in which the consensus site targeted by p53 is in frame with the luciferase reporter gene (PG13), we show that WT-DJ-1 reduces by about 50% the transcriptional activity of p53 while both D149A and L166P mutations abolished this phenotype (Fig.2A, left panel). Accordingly, WT-DJ-1 but not mutated DJ-1 decreases p53 promoter transactivation (Fig.2A, middle panel) and mRNA levels (Fig.2A, right panel). In order to establish whether the anti-apoptotic phenotype of DJ-1 was fully dependent of p53, we took advantage of two cell models in which either $p19^{arf}$ or $p19^{arf}$ and *p53* genes had been invalidated. The depletion of $p19^{arf}$ avoids cell senescence and allows the analysis of the function of p53 in the control of cell death but not in that of cell cycle (Weber et al., 2000). First, it must be noted that both $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts respond to STS and 6OHDA by an activation of their endogenous caspase-3 although to a lesser extent in the latter cell system (Fig.2B, left histogram). Transient transfection of WT-DJ-1 cDNA in the two cell lines reduces STS (grey bars) and 6OHDA (black bars)-stimulated caspase-3 activation by about 40% and 60%, respectively. Here again, the D149A and L166P DJ-1 mutations abolished this phenotype (Fig.2B, left histogram). Interestingly, WT-DJ-1-associated reduction of caspase-3 activation appears totally prevented by p53 deficiency (Fig.2B, right histogram), indicating that DJ-1 protective function is fully dependent of p53.

We examined the mechanisms by which DJ-1 could control p53. Several putative upstream p53 modulators including the PI3 kinase/Akt and extracellular regulated kinases (ERKs) have been described. Thus, the Akt survival pathway was recently shown to down-regulate p53 (Oren, 1999; Brooks and Gu, 2003) by triggering its Mdm2-mediated ubiquitination and subsequent proteasomal degradation (Mayo and Donner, 2001; Ogawara et al., 2002) while ERK belongs to the mitogen-activated protein kinases (MAPK) superfamily that targets p53 (McCubrey et al., 2007). Fig.3A (histogram) shows that the Akt inhibitor LY294002 fully impairs the DJ-1 associated protection against 6OHDA in SH-SY5Y neuroblastoma cells. Conversely, ERK-1 deficiency does not affect the DJ-1-associated phenotype in fibroblasts (Fig.3B). This data clearly suggest that the Akt but not the ERK survival pathway was mainly involved in the DJ-1 associated protective function. Two lines of data led us to examine whether NFκB could mediate DJ-1-associated Akt-dependent regulation of p53. First, two recent studies indicated that there could exist an Akt-dependent phosphorylation of the IκB-kinase that ultimately led to NFκB activation (Milne et al., 2004; Jeong et al., 2005). Second, it was recently demonstrated that several cell systems inhibit p53 activity and enhance cell survival by an Akt-dependent activation of NFκB (Ozes et al., 1999). In this context, we examined whether the pharmacological targeting of the NFκB pathway could influence DJ-1 associated function. Fig.1 suppl. shows that the IκB-kinase inhibitor BMS-345541 does not affect the DJ-1-associated reduction of 6OHDA-induced caspase-3 activation. Overall, the above data demonstrate that DJ-1 modulates p53 by an Akt-dependent but ERK- and NFκB-independent pathway.

That DJ-1 protective function was totally blocked by LY294002 clearly suggested a fully post-transcriptional control of p53 by Akt but was, at first sight, difficult to reconcile with our observation that Akt also down-regulated p53 promoter transactivation. One explanation could be that the post-transcriptional Akt-dependent modulation of p53 could

subsequently trigger a decrease of p53 promoter transactivation by p53 itself. In order to examine this possibility, we have transfected p53 and p53 promoter cDNAs in $p19^{arf-/-}p53^{-/-}$ fibroblasts. In this cell system lacking endogenous p53, any putative activation of p53 promoter could only derive from the functional interaction between transfected p53 promoter and p53. Indeed, Fig.3C shows that p53 over-expression activates its own promoter transactivation in $p19^{arf-/-}p53^{-/-}$ fibroblasts.

In order to investigate if the modulation of p53 by DJ-1 was also linked to additional post-transcriptional events, we have analyzed the ability of DJ-1 to affect the cellular localization of p53. As shown in Fig.2C, the over-expression of WT-DJ-1 increases p53-like immunoreactivity into the cytosol and prevents its nuclear localization while both DJ-1 mutations exacerbate p53 nuclear expression. The export of p53 from nucleus to the cytosol is considered as an inactivating process since it enables p53 ubiquitination by Mdm2 and subsequent degradation by the proteasome (Moll et al., 1992). That WT-DJ1 apparently increased cytosolic localization of p53 could be the consequence of the previous documented chaperoning properties of DJ-1 (Shendelman et al., 2004) and could suggest a putative physical interaction between these two proteins. Fig.2D shows that endogenous WT-DJ-1 can physically interact with p53. This interaction also occurs between over-expressed WT-DJ-1 and p53 and was abolished by pathogenic mutations (data not shown).

Depletion of endogenous DJ-1 increases p53 in mouse fibroblasts and in mice brain.

In order to further establish the role of WT-DJ-1, we examined the contribution of endogenous DJ-1 in the control of p53-dependent cell death by means of DJ-1-deficient fibroblasts (Fig.4A, insert). Fig. 4A clearly shows that the lack of DJ-1 drastically increases the STS- and 6OHDA-mediated caspase-3 activation (Fig.4A, left panel) as well as active caspase-3 immunoreactivity (Fig.4A, right panel). It should be emphasized that DJ-1

deficiency also significantly increases caspase-3 activity in non-stimulated conditions (see CT in Fig.4A left). This observation fits perfectly with our demonstration of a significant reduction of caspase-3 activity in control conditions upon WT-DJ-1 cDNA transfection in TSM1 neurons (Fig. 1B). Of most importance, we have established that this phenotype could be rescued by transient transfection of DJ-$1^{-/-}$ fibroblasts by WT-DJ-1 but not D149A- and L166P-DJ-1 cDNA (Fig.4B). DJ-1-deficient fibroblasts also exhibit increased p53 transcriptional activity and mRNA levels (Fig.4C). *p21* is a well known p53 gene target that is implicated in cell cycle control (Xiong et al., 1993). *p21* promoter sequence in frame with luciferase was therefore used as an alternative readout of p53 transcriptional activity. As expected, DJ-1 depletion increases the transcription of *p21* promoter (Fig.4C). Interestingly, brain samples derived from DJ-1-deficient mice also show a mean augmentation of about 40% of p53 expression levels (Fig.4D), indicating that DJ-1 deficiency observed *in vivo* strictly corroborates data observed at a cellular level.

DJ-1 mutations affect DJ-1 protein stability and susceptibility to caspase-6 proteolysis.

Fig.2A,C suppl. clearly shows that the L166P mutation accelerates DJ-1 breakdown (note that L166P-DJ-1 is only detectable by western blot after long-term (L) exposure) while, conversely the D149A mutation stabilizes DJ-1 (Fig.2C, suppl.). As previously suggested (Miller et al., 2003), over-expressed WT-DJ-1 and L166P-DJ-1 catabolism is prevented by lactacystin and ALLN (Fig.3A,B suppl.) treatments, suggesting a preferential breakdown of these proteins by the proteasomal machinery. Time-courses of DJ-1 expression decay, in presence and absence of cycloheximide, confirm that the two DJ-1 mutations differently affect the stability of the neo-synthesized proteins (Fig.2C, suppl,). Therefore intriguingly, L166P and D149A DJ-1 mutations both lead to a loss of function of p53-dependent control of cell death by DJ-1 in spite of exhibiting clearly distinct catabolic fates. If it appeared

reasonable to propose that the L166P-DJ-1-associated loss of function reflect the low levels of the proteins undergoing rapid proteolysis, such exacerbated instability could clearly not account for the loss of function associated to the degradation-resistant D149A-DJ-1. Interestingly, *in silico* analysis of the human DJ-1 sequence revealed that this protein harbored two putative caspase-6 consensus domains in positions 149 ($VEKD^{149}$) and 60 ($SLED^{60}$). It is well recognized that the aspartyl (D) residue embedded in these consensus sequences is a crucial structural element strictly required for recognition/catalysis of proteins targeted by caspases (Villa et al., 1997). Therefore, the pathogenic D149A mutation described above potentially impairs a caspase-6 cleavage site of DJ-1. In this context, we designed by site-directed mutagenesis a series of constructs either bearing single, double or triple mutations (see nomenclature in table 1 suppl.) and we analyzed the susceptibility of the corresponding proteins to proteolysis by various recombinant caspases. Fig.5B shows that WT-DJ-1, D60A-DJ-1 and L166P-DJ-1 behaved as substrates of caspase-6 but not caspases 3 and 7. Interestingly, D149A-DJ-1 fully resisted proteolysis by caspase-6 (Fig.5B) while the L166P mutation apparently enhanced DJ-1 susceptibility to recombinant caspase-6 (Fig.2B suppl.). Analysis of double and triple mutations confirmed that the proline substitution at position 149 fully blocked the cleavages of 60/149DJ, 149/166DJ and tmutDJ by caspase-6 while 60/166 remained cleaved by recombinant caspase-6 (Fig.5C). These data demonstrate that DJ-1 is cleaved by caspase-6 at only one out of the two putative caspase-6 consensus sequences located at position 146-149.

The caspase-6-derived C-terminal product of DJ-1 accounts for DJ-1-associated protective phenotype.

A question remained as to whether the loss of function triggered by the D149A mutation could be fully due to the impairment of DJ-1 proteolysis by caspase-6. If true, one

would predict that one of the caspase-6 derived proteolytic fragments of DJ-1 should bear its protective function. We have designed, by site-directed mutagenesis, the N-terminal (N-Cas) and C-terminal (WTC-Cas) fragments of WT-DJ-1 theoretically derived from its cleavage after residue 149. Furthermore, we have obtained the caspase-6-derived fragment of L166P-DJ-1 referred to as 166C-Cas (see Table 1 suppl.). Fig.6A shows the expression profile of the proteins derived from these constructs after transient transfection in HEK293 cells. In agreement with previous considerations, we observed a rather poor expression of 166C-Cas (Fig.6A). This prompted us to analyze 166C-Cas mRNA levels in order to rule out the possibility that distinct transfection efficiencies could interfere with interpretation of the data. Fig.6B shows that mRNA levels were similar in all transfection experiments. Therefore, the L166P mutation indeed drastically enhances the catabolic instability of both entire and C-terminal fragments. It should be emphasized that the WTC-Cas fragment was also recovered at lower levels than the N-terminal counterpart (Fig.6A), suggesting that the stability of DJ-1 was clearly due to its C-terminal moiety. This agrees well with the fact that DJ-1 homodimerization was reported to occur through the association of the C-terminal part of the proteins and that the L166P impairs such association (Moore et al., 2003).

Fig.6C shows that transient transfection of HEK293 cells with the WTC-Cas cDNA triggers a drastic reduction of caspase-3 activity in non-stimulated conditions (empty bars) as well as in STS- (grey bars) or 6OHDA- (black bars) stimulated conditions while N-Cas remained totally inactive. Interestingly, the WTC-Cas-mediated response was abolished by the L166P mutation (Fig.6C). This phenotype was further confirmed by the analysis of the levels of active caspase-3 in the same transfected samples (Fig.6D). Of most interest, WTC-Cas was also the only fragment of DJ-1 able to lower p53 transcriptional activity (Fig.6E). Of importance, the protective effect associated to WTCcas was also observed in primary cultured neurons (Fig.6F). Finally, we confirmed the protective function harbored by the WTC-Cas by

complementation experiments in DJ-1-deficient fibroblasts. Fig.6G illustrates the influence of transient transfection of DJ-$1^{-/-}$ fibroblasts by WTDJ, N-Cas, WTC-Cas and 166C-Cas. We show that only WTDJ and its caspase-6-derived fragment WTC-Cas similarly reduced caspase-3 activation while N-Cas and 166C-Cas remained biologically inactive. Overall, our data demonstrate that the caspase-6-derived C-terminal product of DJ-1 fully accounts for the p53-dependent control of caspase-3 by DJ-1.

Altered processing of DJ-1 in sporadic PD brains

In order to examine whether our cellular data could be extrapolated to a physiological context, we analyzed the levels of DJ-1 and caspase-6 in human brains obtained from control and sporadic PD patients. As depicted in Fig.7A,B, the levels of caspase-6 and DJ-1 appeared inversely correlated in normal and PD-affected brains, suggesting that increased expression of caspase-6 activity could explain the slight but statistically significant reduction of DJ-1 levels observed in pathological brains.

Discussion

Several studies have consistently documented a protective role of DJ-1 against various pro-apoptotic effectors or stress-inducing agents (Alves da Costa, 2007) but the mechanisms underlying such function still remained barely known. Here we demonstrate that the over-expression of DJ-1 lowers STS- and 6OHDA-induced caspase-3 activation via a reduction of p53 expression and activity in various cell systems including primary cultured neurons, TSM1 neurons, SH-SY5Y neuroblastoma, HEK293 cells and mouse fibroblasts. Of most importance, the depletion of endogenous DJ-1 triggers an opposite phenotype that can be rescued by complementation of these cells with the wild-type DJ-1 construct. To our knowledge, this is the first demonstration that DJ-1 reduces the p53 pathway in human and

neuronal cells in response to various stimuli, including 6OHDA, a naturally occurring toxic dopaminergic by-product (Jellinger et al., 1995). This data agrees well with another work showing that DJ-1 depletion triggers increased Bax and p53 expressions in the zebrafish (Bretaud et al., 2007).

Two distinct lines of data indicate that the DJ-1-associated phenotype is fully dependent of p53 and involves post-transcriptional control of this tumor suppressor. First, DJ-1-associated protective function is fully abolished by p53 deficiency. Second, DJ-1 controls p53 by activating Akt-dependent and Erk and NFκB-independent pathways and by triggering its sequestration within the cytosol. This data agrees well with a previous study documenting the fact that down-regulation of DJ-1 levels by siRNA targeting approaches lead to reduced Akt phosphorylation, *in vivo* (Yang et al., 2005). Interestingly, DJ-1 also lowers p53 promoter transactivation. This appeared paradoxical with respect to our data showing that the pharmacological blockade of the Akt pathway fully prevents DJ-1 associated phenotype. However, in agreement with previous data (Deffie et al., 1993), our demonstration that p53 could activate its own transcription in fibroblasts (Fig.3C) suggests that this phenomenon could likely account for DJ-1-induced decrease of p53 promoter transactivation.

Although most of the cases of PD are of sporadic origin, it remains that the delineation of the molecular dysfunctions responsible for a rather low subset of genetic cases should help understanding PD pathogenesis. To date, only one deletion and one point mutation in the gene of DJ-1 have been reported in two distinct families. In the latter case, the replacement of a leucine residue in position 166 by a proline introduces a strong helix breaking element that precludes DJ-1 homodimerization, (Moore et al., 2003) increases catabolic instability and thereby, impairs it biological activity. More recently, another mutation that replaces an aspartyl residue in position 149 by an alanine was reported to trigger early onset PD although

the family history is not yet available to ascertain that transmission of this mutation results from a recessive transmission.

At a macroscopic level, the two DJ-1 mutations led to similar impairment of DJ-1-mediated anti-apoptotic phenotype. Thus, both L166P and D149A mutations abolish DJ-1-induced reduction of STS- and 6OHDA-stimulated caspase-3 activity and were unable to rescue DJ-1 phenotype in complementation experiments performed in DJ-1-deficient fibroblasts. On the other hand, it clearly appeared that unlike D149A-DJ-1 that behaves as the parent protein, L166P-DJ-1 displayed a remarkable short life in our cells. The above observations suggested that mutations at residues 149 and 166 similarly impair DJ-1 protective function through distinct mechanisms.

The fact that an aspartyl residue substitution was apparently responsible for DJ-1 dysfunction led us to envision that the D149A mutation could have prevented DJ-1 catabolism by either aspartyl proteases or cystein caspase-like activities. Indeed, *in silico* analysis of DJ-1 sequence identified two consensus sites for caspase-6 activity corresponding to SLED and VEKD tetrapeptides where aspartyl residues were in positions 60 and 149, respectively. Thus, we envisioned the possibility that the D149A mutation could impair DJ-1 proteolysis and, as a corollary, that such resistance to caspase-6 mediated cleavage could be responsible for the loss of function triggered by the mutation. Four distinct lines of evidence support this hypothesis. First, DJ-1 was readily proteolysed by recombinant caspase-6 but not caspases-3 and -7. Second, the D149A but not the D60A mutation fully impaired DJ-1 cleavage by caspase-6. Third, the C-terminal fragment of DJ-1 (WTC-Cas) that would theoretically derive from its processing by caspase-6 at site 149 mimics DJ-1 phenotype, reduces STS- and 6OHDA-stimulated caspase-3 activity and lowers p53 transcriptional activity. Fourth, WTC-Cas fully rescues DJ-1 phenotype after complementation experiments in DJ-1 deficient fibroblasts. Altogether, these data demonstrate that the C-terminal moiety of DJ-1

corresponding to WTC-Cas is responsible for DJ-1-associated protective phenotype and that this function is abolished by the D149A mutation that impairs caspase-6-mediated cleavage of DJ-1. Thus, our study is the first demonstration that DJ-1 anti-apoptotic function is controlled by its proteolysis by caspase-6. It is interesting to note that synphilin-1, a physiological partner of α-synuclein (Engelender et al., 1999) also undergoes caspase-mediated biological maturation. Thus, we established recently that synphilin-1 is readily targeted by caspase-3 and poorly processed by caspases-6 and -7 (Giaime et al., 2006). Interestingly, the C-terminal fragment of synphilin-1 generated by caspase-3 harbored a protective function by controlling the p53-dependent pathway (Giaime et al., 2006). Overall, this data and our present work reinforced the current view that the p53-dependent cell death could be a central process in PD pathology (Blum et al., 1997; Duan et al., 2002) but also unmask the crucial involvement of caspase-dependent proteolytic events in the function of PD-associated proteins and suggest that distinct caspases could be specifically involved in the physiological maturation of these proteins. On the other hand, caspase-mediated cleavages could be seen as inactivating mechanisms. Thus, parkin undergoes proteolysis by caspases 1 and 8 (Kahns et al., 2003) leading to an impairment of its ubiquitin-like domain (UBL). This process inactivates the parkin-associated E3-ligase activity that controls levels of ubiquitinated CDCrel-1 and Pael-R (Dong et al., 2003; Yang et al., 2003) and therefore their subsequent proteasomal degradation. Overall, the above studies along with our present work reveal the crucial involvement of caspase-dependent proteolytic events in the function of PD-associated proteins and suggest that distinct caspases could be specifically involved in the physiological maturation and/or inactivation of these proteins.

Our study is also the very first report demonstrating that various pathogenic mutations could trigger DJ-1 loss of function via totally distinct molecular mechanisms, i.e protein destabilization or resistance to caspase-6-mediated proteolysis (see Fig.8). In line with our

data, a recent study also indicates that parkin mutations impair its E3-ligase activity by triggering either misfolding or destabilization of the protein (Henn et al., 2005). The loss of the E3-ligase activity likely results in an impairment of parkin function since the deletion of the UBL domain of parkin compromises its ability to protect cells against ceramide-induced toxicity (Darios et al., 2003).

Of most interest, we have established that the levels of endogenous caspase-6 in normal and sporadic PD were inversely correlated (see Fig.7). This strongly corroborates our view that caspase-6 is indeed a physiological modulator of endogenous DJ-1 levels in human brain. Whether the increased caspase-6 expression observed in sporadic PD could be seen as a putative compensatory mechanism aimed at interfering with the PD-associated degenerative process remains to be established. However, it is tempting to suggest that pathogenic mutations differently affecting caspase-6-mediated proteolysis of DJ-1 could lead to distinct onsets and phenotypes as it was observed for Parkinson's disease patients harboring distinct parkin mutations (Henn et al., 2005).

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Competing interests statement The authors declare that they have no competing interests.

Legends

Figure 1. DJ-1 lowers staurosporine-induced caspase-3 activation in human and murine neuronal cells: effect of PD-associated mutations.

(A) TSM1 cells were stably transfected with the indicated cDNA then V5-tagged DJ-1 and actin expressions (A, upper panel) and mRNA levels (A, lower panel) were analyzed by western blot and real time PCR, respectively as described in the "Experimental Procedure". Bars correspond to mRNA levels expressed in arbitrary units and are the means ± S.E.M of three independent experiments performed in triplicates. (B,C) The indicated TSM1 cell lines were treated for 8 hours with vehicle (empty bars) or with 6OHDA (0.2 mM, black bars) then caspase-3 activity (B) and poly-ADP-ribose polymerase cleavage (C) were analyzed as described in the "Experimental Procedure". Bars in B are the means ± S.E.M of 9-10 independent experiments performed in duplicates. *$p<0.05$; ***$p<0.001$, ns, not statistically significant. (D) SH-SY5Y human neuroblastoma cells were transiently transfected with the indicated cDNA. Twenty-four hours after transfection, cells were treated for 8 hours without (empty bars) or with 6OHDA (0.2mM, black bars) then caspase-3 activity was monitored as described in the "Experimental Procedure". Bars are the means ± S.E.M of 4-5 independent experiments performed in duplicates. *$p<0.05$; **$p<0.01$, ns, not statistically significant.

Figure 2. PD-associated mutations abolish DJ-1-induced down-regulation of the p53 pathway.

(A) The indicated TSM1 cell lines were monitored for their p53 activity (PG13), promoter transactivation (mpp53) and mRNA levels (mRNA) as described in the "Experimental Procedures". Bars correspond to the means ± S.E.M of 3-4 independent experiments performed in 3-6 replicates. *p<0.05; ***p<0.001. ns, not statistically significant. (B) $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts were transiently transfected with the indicated cDNA. Twenty-four hours after transfection, cells were treated for 2 hours with STS (2μM) or 8 hours with 6OHDA (0.2mM) then expression of proteins (B, upper panels) and caspase-3 activity (B, lower panels) were monitored as described in the "Experimental Procedure". Bars correspond to the means ± S.E.M of 3-5 independent experiments. ***p<0.001. ns, not statistically significant. (C) Nuclear and cytoplasmic expression of p53 was analyzed in the indicated stably transfected TSM1 cell lines as described in the "Experimental Procedure". (D) Physical interaction between endogenous DJ-1 and p53. DJ-1 was immunoprecipitated (IP) with anti-DJ-1 antibody then p53- and DJ-1-like immunoreactivities were analyzed by western blot as described in the "Experimental Procedure". Note that IP of DJ-1 traps both DJ-1 and p53.

Figure 3. DJ-1 modulates p53 at a post-transcriptional level.

(A) SH-SY5Y neuroblastoma cells were transiently transfected with the indicated cDNA. Twenty-four hours after transfection, cells were treated with either LY294002 (10μM, 16 hours), 6OHDA (0.2mM, 8 hours) or both compounds then analyzed for DJ-1 and actin expressions (upper panels) and for caspase-3 activity as described in the "Experimental Procedure". Bars correspond to the means ± S.E.M of 4 independent experiments performed in duplicates. **p<0.01. ns, not statistically significant. (B) Wild-type (left panel) and ERK-deficient (right) fibroblasts were transiently transfected with empty $pcDNA_3$ vector (black bars) or wild-type DJ-1 (empty bars) cDNAs. Twenty-four hours after transfection, cells were treated without (-) or with (+) 6OHDA (0.2mM, 8 hours) then DJ-1 expression (upper panel) and caspase-3 activity were monitored as described in the "Experimental Procedure". Bars correspond to the means ± S.E.M of 6-8 independent experiments performed in duplicates. ***p<0.001. (C) $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts were transiently co-transfected with the mouse p53 promoter in combination with either empty $pcDNA_3$ or p53 cDNA. p53 promoter transactivation was monitored as described in the "Experimental Procedure". Bars correspond to the means ± S.E.M of 5 independent experiments. ***p<0.001.

Figure 4. DJ-1 deficiency alters the p53 pathway in fibroblasts and in mice brain.

(A) Wild-type (empty bars) and DJ-1 deficient fibroblasts (grey bars) were treated with STS (2μM, 2 hours) or with 6OHDA (0.2mM, 8 hours) then active caspase-3 expression (right panel) and caspase-3 activity (left panel) were measured as described in the "Experimental Procedure". Bars correspond to the means ± S.E.M of 3-6 independent experiments. *$p<0.05$; ***$p<0.001$. ns, not statistically significant. (B)

DJ-1-deficient fibroblasts were transiently transfected with the indicated cDNA as described in the "Experimental Procedure". Twenty-four hours after transfection, cells were treated for 8 hours with vehicle (white bars) or with 6OHDA (0.2mM, black bars) then caspase-3 activity was monitored as above. Bars correspond to the mean of ± S.E.M of 12-16 independent experiments. **$p<0.01$. ns, not statistically significant. (C) Wild-type and DJ-1-deficient fibroblasts were monitored for their p53 activity (PG13) and mRNA levels (mRNA) as well as for the transactivation of the promoter of p21 as described in the "Experimental Procedure". Values are expressed as the percent of control activities obtained in wild-type fibroblasts (taken as 100) and are the means ± S.E.M of 3-4 independent experiments performed in 6 (PG13 and p21) or 3 (mRNA levels) replicates. *, $p<0.05$; ***, $p<0.001$.

(D) Expression patterns (left) and densitometric analyses (right) of endogenous DJ-1 and p53 in wild-type ($DJ\text{-}1^{+/+}$) and DJ-1 knockout ($DJ\text{-}1^{-/-}$) mice brain extracts were measured as described in the "Experimental Procedure".

Figure 5. Distinct susceptibility of wild-type and mutated DJ-1 to proteolysis by caspases.

(A) Localization and nature of the three different pathogenic point mutations on DJ-1. DJ-1 mutants harboring one (60DJ, 149DJ and 166DJ), two (60/149DJ, 60/166DJ and 149/166DJ) or three (tmutDJ) mutations (for nomenclature see table 1 in supplementary materials) were obtained by site-directed mutagenesis as described in the "Experimental procedure" (A, alanine; D, aspartic acid; L, leucine; P, proline). (B-C) The indicated constructs were transcribed and translated *in vitro* with (Shang et al.)methionine and incubated for 8 hours at 37°C with purified recombinant caspase-3, -6, and -7 (C3, 6, 7; 25ng) in absence or presence of caspase inhibitor (Ac-DEVD-CHO ,10μM).

Figure 6. The caspase-6-derived C-terminal fragment of DJ-1 lowers caspase-3 activation and p53 activity.

(A-B) HEK 293 cells were transiently transfected with the indicated cDNA (see table 1, supplementary material). Expression (A) and mRNA levels (B) of DJ-1 fragments were analyzed by western blot with anti-V5 antibodies (A) or by RT-PCR with V5-specific primers (B) as described in the "Experimental procedure". (C-D) HEK293 cells were transiently transfected with the indicated cDNA. Twenty-four hours after transfection, cells were treated with STS (2μM, 16 hours, grey bars) or with 6OHDA (0.2mM, 8 hours, black bars) then caspase-3 activity (C) and active caspase-3 immunoreactivity (D) were monitored as described in the "Experimental Procedure". Bars in C represent the means ± S.E.M of 4-5 independent experiments performed in duplicates and are expressed as percent of control caspase-3 activity obtained in untreated mock-transfected cells. **, $p<0.01$ (comparison between WTC-Cas versus Mock, N-Cas, and 166C-Cas). ns, not statistically significant. (E) HEK 293 cells were co-transfected with PG13-luciferase cDNA and the indicated cDNA then p53 activity was measured as described in the "Experimental Procedures". Bars correspond to the means ± S.E.M of 4 independent experiments performed in 6 replicates. *, $p<0.05$; **$p<0.01$. ns, not statistically significant. (F) Primary neurons were transiently transfected by nucleoporation with the indicated cDNAs as described in "Experimental Procedures". Four days after transfection, neurons were treated for 16 hours with STS (2μM) then caspase-3 activity was monitored as described in "Experimental Procedures". Bars are the means ± SEM of 5 independent experiments corresponding to the STS-stimulated caspase-3 activity. **$p<0.01$. ns, not statistically significant. (G) DJ-1-deficient cells were transiently transfected by nucleoporation with the indicated cDNA as described in "Experimental Procedures". Twenty-four hours after transfection, cells were treated for 8 hours with vehicle (white bars) or with 6OHDA (0.2mM, black bars) then caspase-3 activity was monitored as above. Bars correspond to the means ± S.E.M of 10-16 independent experiments and are expressed as percent of control caspase-3 activity obtained in mock-transfected fibroblasts. **$p<0.01$. ns, not statistically significant

Figure 7. Caspase-6 and DJ-1 levels are altered in the substantia nigra of sporadic Parkinson's disease -affected human brains.

Caspase-6 and DJ-1 expressions (A) and densitometric analyses (B) in control (CT, triangles, see Experimental Procedure) and sporadic Parkinson's disease (PD, dots) human brains were analyzed as described in the "Experimental Procedures". Each point in B corresponds to 3 independent determinations. *$p<0.05$.

Figure 8. Model of DJ-1-associated cell protection and its alteration by pathogenic mutations.

Caspase-6 activation by a cellular stress triggered by staurosporine or the natural dopaminergic toxin 6OHDA increases caspase-6-mediated cleavage of wild-type DJ-1, thereby generating a C-terminal DJ-1 fragment that activates Akt, reduces p53 activity and therefore acts as a protective cellular signal (blue pathway). Mutation of DJ-1 at amino-acid 149 prevents its cleavage by caspase-6. A DJ-1 mutation at position 166 destabilizes DJ-1 and renders the protein prone to rapid degradation. Both mutations trigger a loss of function of DJ-1 and abolish DJ-1 mediated control of p53 (red pathway). Therefore, various mutations of DJ-1 can be likely responsible for Parkinson's disease pathology via distinct molecular dysfunctions.

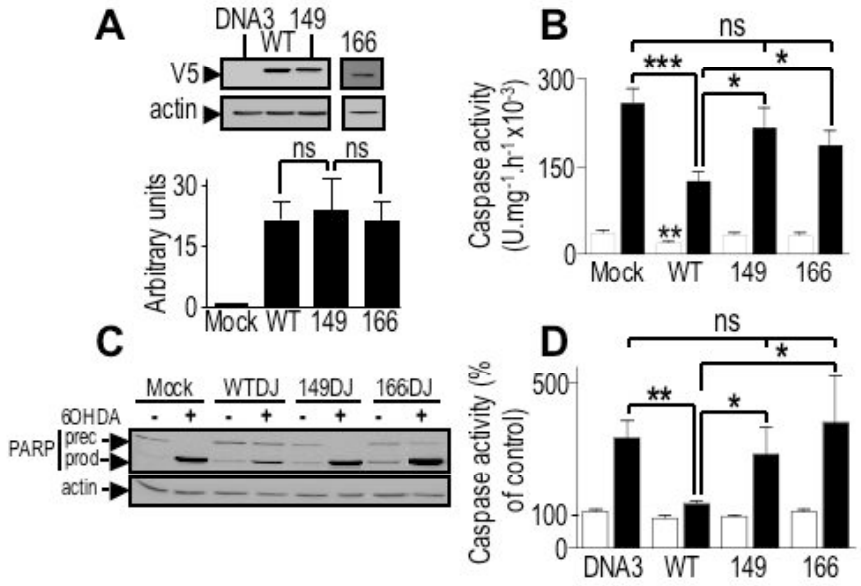


Figure. 1 Giaime et al.

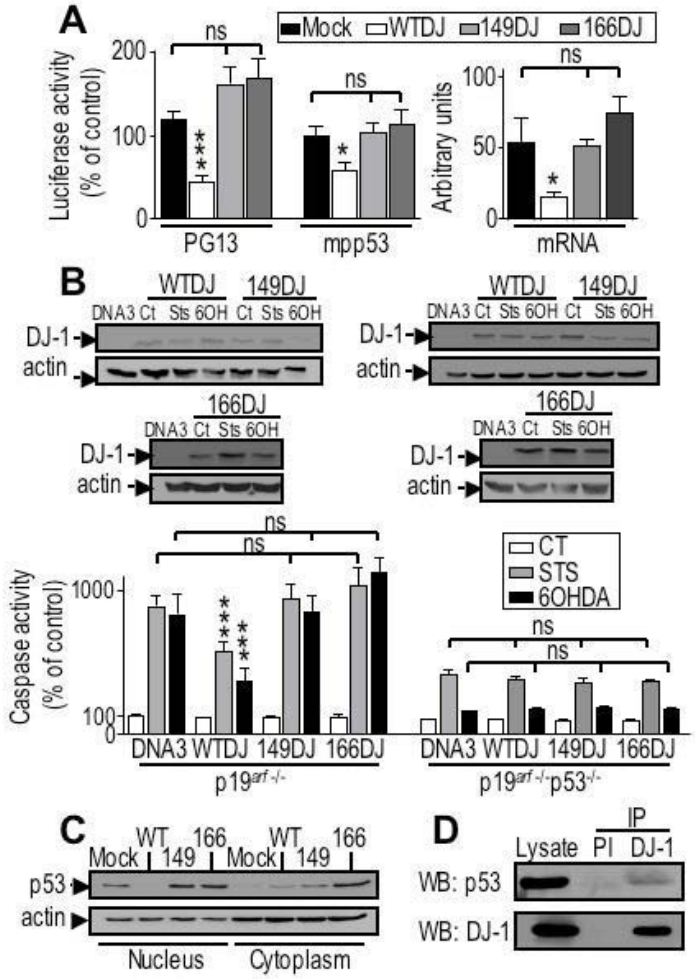


Figure. 2 Giaime et al.

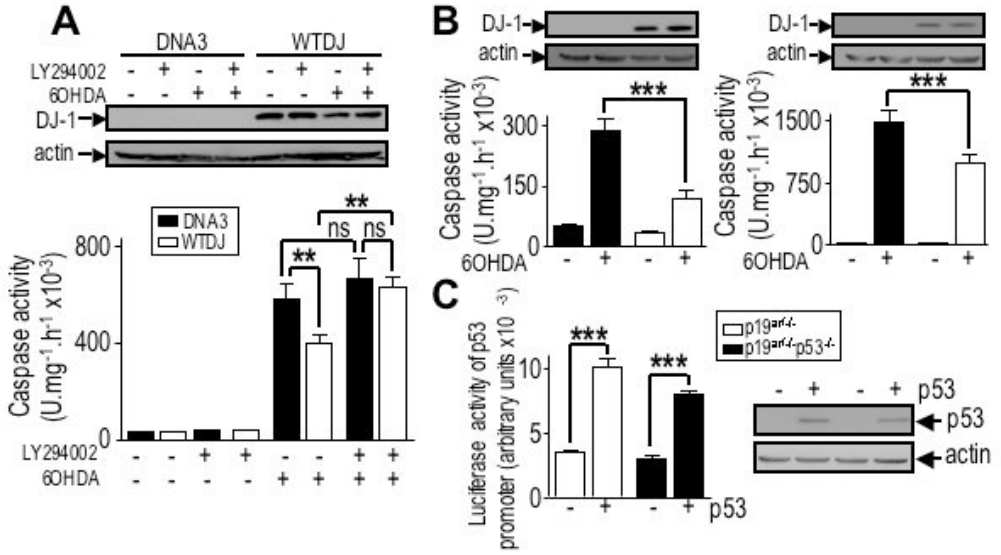
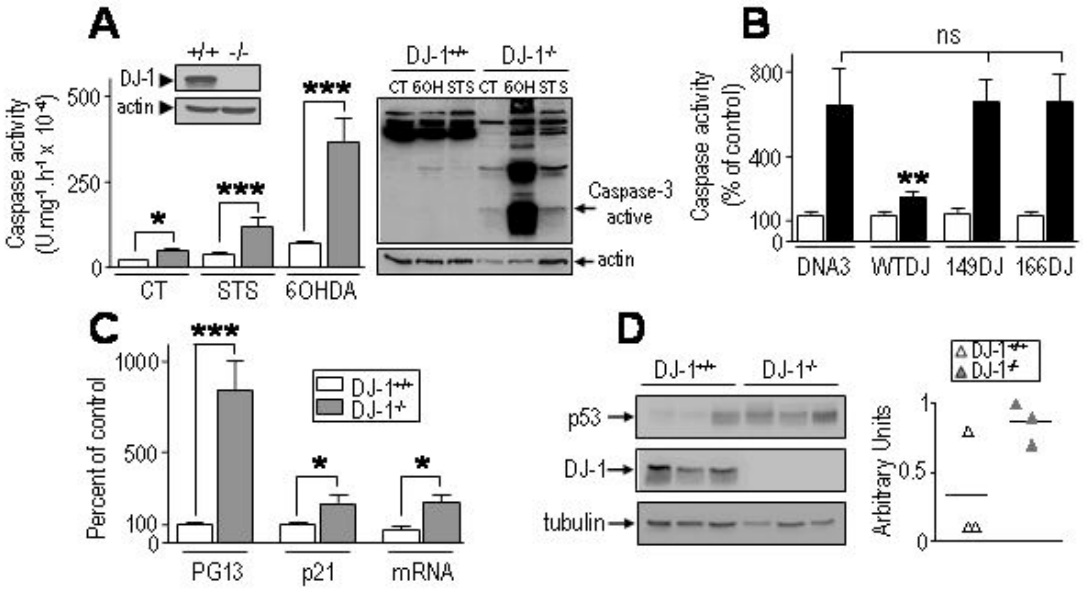


Figure. 3 Giaime et al.



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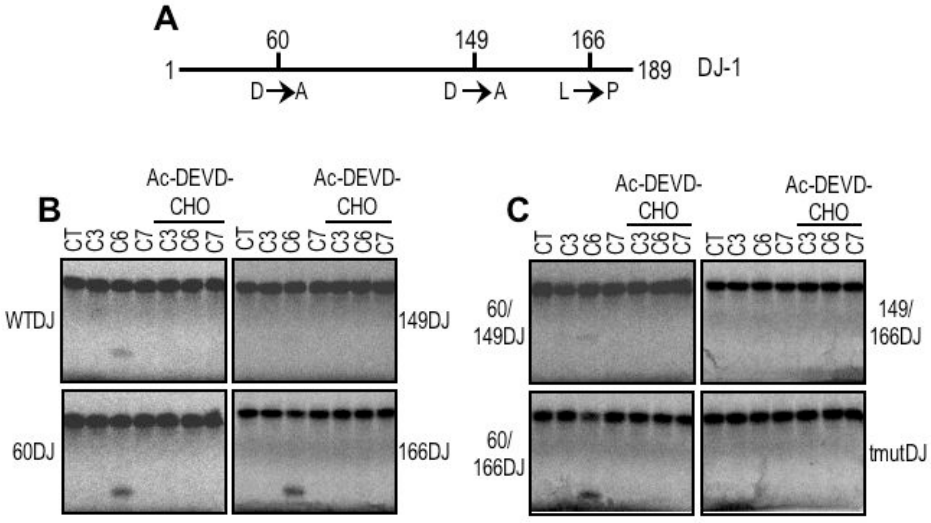
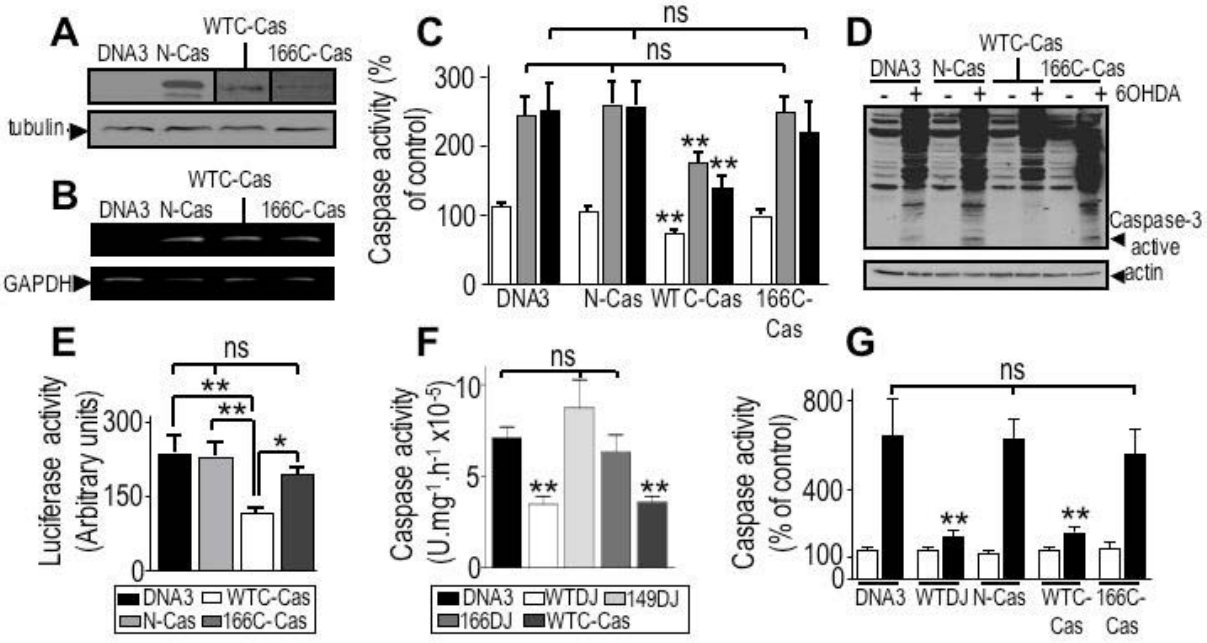


Figure. 5 Giaime et al.



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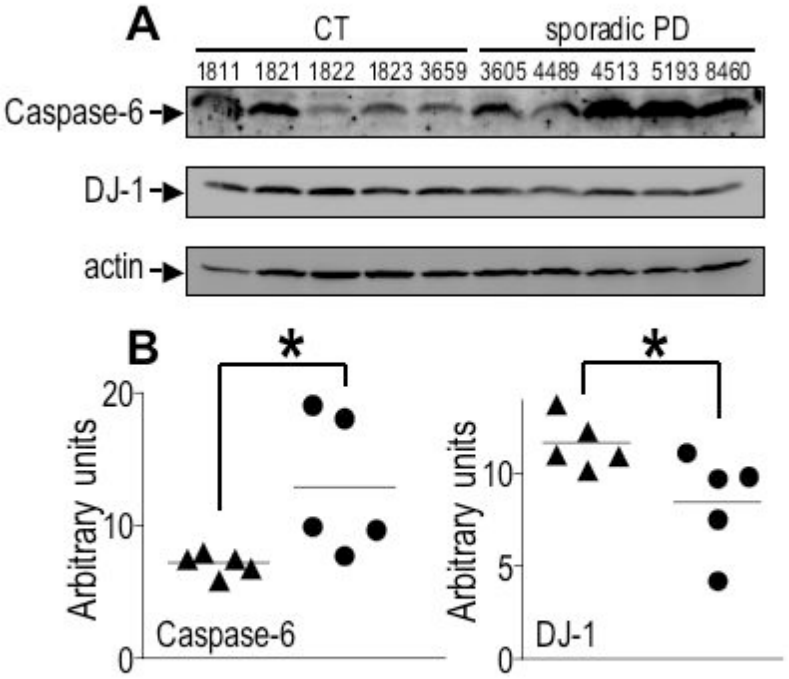

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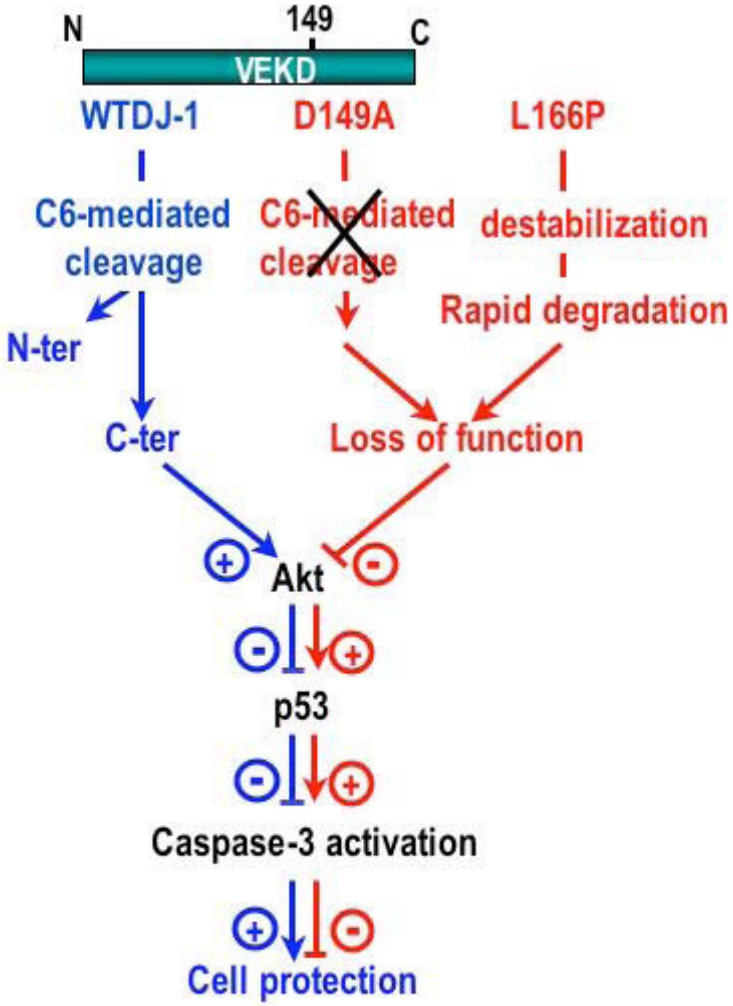

Figure. 8 Giaime et al.

B.III) La Parkine régule p53 au niveau transcriptionnel (Article 3)

Article 3: "Transcriptional repression of p53 by parkin and impairment by Parkinson's disease-associated mutations." Soumis

La fonction protectrice de la parkine est associée à une modulation de p53.

Dans cette étude nous avons généré une lignée cellulaire issue de TSM1 surexprimant stablement la parkine sauvage. Si l'on soumet ces cellules à un traitement à la STS et à la 6-OHDA on observe une diminution de l'activité caspase-3 induite par cette stimulation dans les cellules exprimant la parkine sauvage. Dans ces cellules l'on observe également une diminution de la voie p53 (expression, activité, taux d'ARNm, transactivation du promoteur), cette diminution de l'expression de p53 est également observable dans des cultures primaires de neurones corticaux embryonnaires. De plus, la transfection de la parkine dans des cellules dépourvues de p53 nous a permis de déterminer que cette fonction protectrice de la parkine est dépendante de la présence de p53 (Figure 1).

En accord avec ces données dans des cellules transfectées, lorsque l'on traite des cellules fibroblastiques de souris (MEF) invalidées pour le gène de la parkine à la 6-OHDA, on observe une augmentation de l'activité caspase-3. Dans ces cellules la voie p53 est aussi augmentée (expression, activité, taux d'ARNm), tout comme dans des cerveaux de souris invalidées pour la parkine. La complémentation de ces cellules avec l'ADNc de la parkine entraîne une réversion du phénotype induit par l'invalidation (Figure 2).

Des mutations pathogènes sur le gène de la parkine entraînent une réversion de la fonction protectrice de celle-ci.

La transfection transitoire dans des cellules SH-SY5Y d'ADNc de la parkine contenant des mutations inhibant son activité ubiquitine ligase (C418R et C441R) ou non (K161N et R256C), inverse la fonction protectrice de la parkine. En effet, la parkine mutée n'est plus capable de diminuer l'activité caspase-3 induite par un

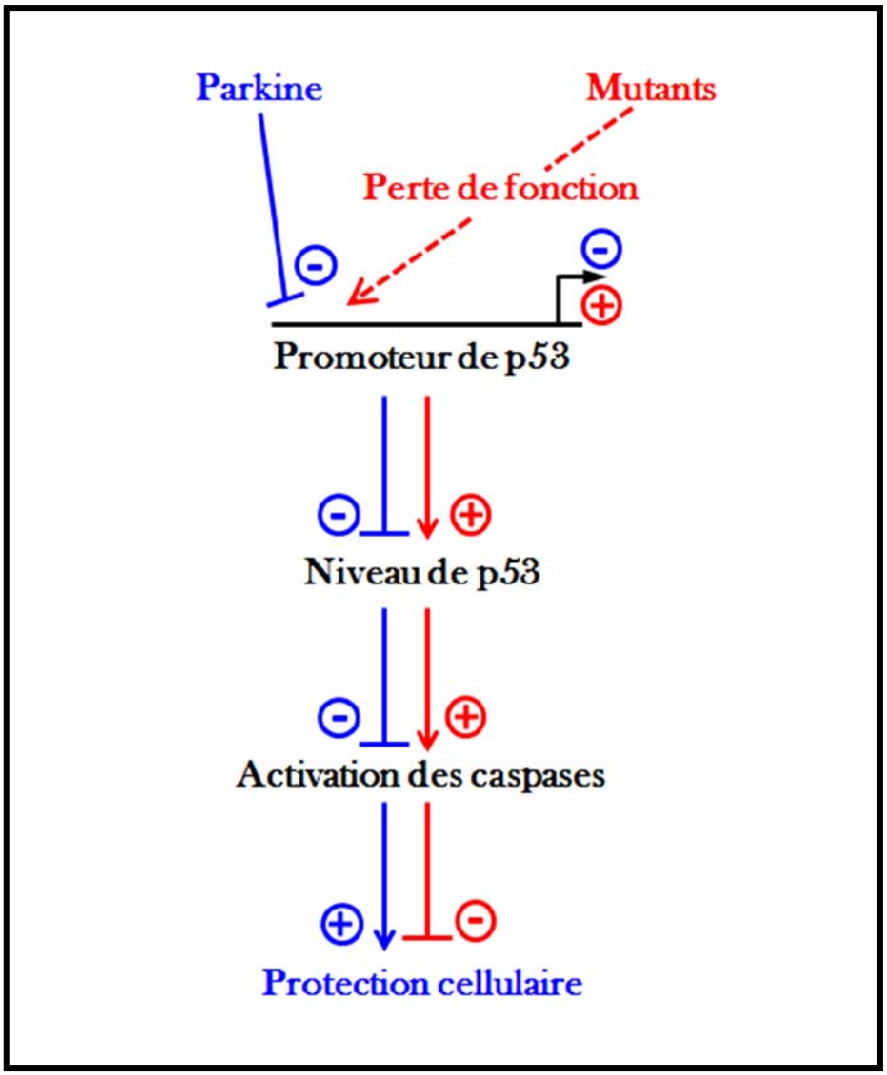

Figure 40 : Schéma récapitulant les mécanismes impliqués dans la fonction protectrice de la parkine

traitement à la 6-OHDA. Elle n'inhibe plus non plus l'expression et la transactivation du promoteur de p53. La complémentation de cellules déficientes en parkine avec l'ADNc de la parkine mutée ne permet pas de inverser le phénotype induit par cette invalidation. De plus, *in vivo,* dans des cerveaux de patients souffrant de formes familiales induites par des mutations sur la parkine, on observe une augmentation de l'expression de p53 (Figure 3).

La parkine régule la voie p53 à un niveau transcriptionnel.

L'utilisation d'un inhibiteur de la voie Akt (LY294002) (Figure Suppl. 2) a permis de déterminer que la parkine ne module pas l'apoptose en passant par cette voie. Son contrôle sur la voie p53 pourrait donc s'effectuer à un niveau transcriptionnel.

La transfection dans des cellules SH-SY5Y de différents formes du promoteur de p53, plus ou moins tronquées, a permis de mettre en évidence la région du promoteur de p53 avec laquelle la parkine pourrait interagir. Nous avons donc synthétisé des sondes correspondant à différents fragments du promoteur et en particulier à cette région promotrice, que nous avons utilisées dans une expérience de gel retard. Cette expérience montre que la parkine interagit directement avec cette région promotrice afin de réguler la transcription de p53 (Figure 4).

Conclusion/ Discussion.

Ces travaux ont montré que la parkine exerce une fonction protectrice passant étant dépendante de la voie p53. Cette régulation de la voie p53 est assurée par une nouvelle fonction de la parkine. Ces expériences ont donc permis de mettre en évidence une fonction de la parkine encore inconnue à ce jour, à savoir l'activité de facteur de transcription (Figure 40). Il sera intéressant d'identifier d'autres cibles de la parkine, en particulier des gènes impliqués dans les mécanismes d'apoptose.

Article 3

Alves da Costa C., Giaime E., West A., Corti O., Brice A., Abou-Sleiman P.M., Wood N.W., Takahashi H., Goldberg M.S., Shen J., and Checler F.

"Parkin-induced transcriptional repression of p53 is impaired by Parkinson's disease-associated mutations."

En révision

Transcriptional repression of p53 by parkin and impairment by Parkinson's disease-associated mutations

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Key words: Parkinson's disease, familial mutations, parkin, transcription, neuroprotection, p53.

Parkin is an ubiquitin-ligase, mutations of which account for most of autosomal recessive Parkinson's disease cases. Several papers suggested a nuclear localization of parkin and *in silico* studies revealed that parkin contains an IBR domain. Parkin may therefore possess DNA-binding and transcriptional activity but the identification of its putative transcriptional targets was lacking. Here we show that parkin functions as a p53 repressor. Thus, parkin over-expression prevents 6-hydroxydopamine-induced caspase-3 activation in a fully p53-dependent manner. Concomitantly, parkin reduces p53 expression and activity, an effect abrogated by familial parkin mutations known to either abolish or preserve its ligase activity. Parkin physically interacts with p53 promoter, represses its transactivation and lowers p53 mRNA levels. Conversely, parkin depletion enhances p53 expression in both fibroblasts and mice brains and increases cellular p53 activity, promoter transactivation and mRNA levels. Finally, familial parkin missense and deletion mutations enhance p53 expression in Parkinson's-affected human brains. This study reveals a novel ubiquitin-ligase independent function of parkin in the control of transcription and evidences a functional link between parkin and p53 that is altered by familial Parkinson's disease mutations.

Parkinson's disease (PD) is a movement disorder that is characterized by a severe loss of dopaminergic neurons probably dying by apoptosis. This syndrome is mainly idiopathic but about 5% of cases are linked to a Mendelian pattern of inheritance and may be either associated with an autosomal dominant or recessive mode of transmission. Parkin is responsible for the majority of the recessive cases of PD and mutations of this gene are responsible for a particularly aggressive pathology usually characterized by an early onset. Parkin is an ubiquitin-ligase[1] which acts as a negative modulator of apoptosis, both *in vitro* and *in vivo*[2,3]. Since parkin enzymatic activity is abolished by PD-associated mutations, it has been proposed that this functional deficit could account for the observed accumulation of

proteasome-resistant and potentially toxic proteins observed in parkin-related familial cases of PD[1].

Interestingly, several lines of evidence indicate that parkin could also behave as a transcription factor. First, parkin has been shown to be localized in the nucleus [4,5] and it harbors an IBR (for In Between Ring) domain which predicts putative DNA binding and transcriptional activity properties [6]; second, parkin down-regulates the expression of several proteins, the levels of which are enhanced upon apoptotic stimulus [7]. Therefore, whether the cytoprotective effect of parkin was associated with its ability to control proteasomal degradation and cellular homeostasis of a set of cell death-modulators via its ubiquitin-ligase activity or whether this phenotype was linked to a direct or indirect modulation of gene expression remained questionable. It is, however, noticeable that parkin-mediated direct transcriptional control of putative target genes has not been yet documented. Here, we demonstrate that parkin acts as a transcriptional repressor of p53 independently of its ligase function and that familial PD-associated parkin mutations abolish the parkin-mediated control of p53, both *in vitro* and *in vivo*

We have obtained stably transfected TSM1 neurons over-expressing HA-tagged wild-type parkin (Wt-Pk, Fig.1a). Wt-Pk (clone 15, Fig.1b and clone 5, not shown) protects TSM1 neurons from a variety of pro-apoptotic stimuli. Thus, Wt-Pk reduces staurosporine- (STS) and 6-hydroxydopamine (6OHDA)-induced caspase-3 activation by 70% and 84% ($n=10$, $p<0.01$ compared to mock-treated cells), respectively (Fig.1b). Concomitant to the parkin-associated protective phenotype, we have established that parkin controls the p53 pathway at several steps. Thus, over-expression of Wt-Pk induces drastic reductions of p53 expression (p53, 82%, $n=6$, $p<0.001$), transcriptional activity (PG13, 96%, $n=6$, $p<0.0001$), promoter transactivation (Pp53, 56%, $n=6$, $p<0.01$) and mRNA levels (mRNA, 86%, $n=3$, $p<0.01$) when compared to Mock-transfected control cells (Fig.1c). Interestingly, lentiviral over-

expression of Wt-Pk dose-dependently reduces p53 expression in primary cultured neurons (Fig.1d).

We have examined whether parkin-associated reduction of 6OHDA-stimulated caspase-3 activity was strictly dependent of p53. Thus, we compared the effect of parkin over-expression (Fig.1e) in $p19^{Arf\text{-}1\text{-}/\text{-}}$ and $p19^{Arf\text{-}1\text{-}/\text{-}}p53^{-/-}$ fibroblasts. These two cell systems allow examining the effect of p53 on apoptosis without influence of this oncogene on the control of cell cycle [8]. As shown in Fig.1f, parkin reduces 6OHDA-induced caspase-3 activation in $p19^{arf\text{-}1\text{-}/\text{-}}$ cells. Clearly, while caspase-3 remains stimulated by 6OHDA in $p19^{Arf\text{-}1\text{-}/\text{-}}p53^{-/-}$, p53 depletion fully prevented parkin-associated reduction of 6OHDA-induced caspase-3 activation in this cell system (Fig.1f). Overall, this indicates that the control of caspase-3 activity by parkin was strictly p53-dependent.

To examine the implication of endogenous parkin in the control of p53, we have analysed the responsiveness of fibroblasts devoid of parkin (Pk^{-}) to 6OHDA. Parkin depletion leads to a substantial augmentation of caspase-3 activity in both control (163%, n=6, $p<0.05$) and 6OHDA-induced conditions (247%, n=6, $p<0.01$, Fig.2a). Parkin depletion increases p53 expression (231% of control, n=6, $p<0.01$, Fig.2b), activity (632%, n=6, $p<0.01$, Fig.2c), promoter transactivation (275%, n= 6, $p<0.01$, Fig.2c) and mRNA levels (338%, n=5, $p<0.05$, Fig.2c). Of most interest, we establish that brain homogenates prepared from parkin knockout mice also display increased p53 expression (145% of wild-type brain, n=4, $p<0.05$, Fig.2d). Furthermore, we have examined the potential of wild-type parkin to rescue parkin's ability to control p53 in parkin-deficient fibroblasts. First we established that wild-type parkin lowers p53 promoter transactivation in wild-type fibroblasts (35% of reduction, n=6, $p<0.05$, data not shown). Of most interest, transient transfection of wild-type parkin cDNA (Fig.2e) in parkin-deficient fibroblasts rescues parkin's ability to reduce p53 promoter transactivation (Fig.2f)

and mRNA levels (Fig.2g). Overall, our data demonstrate that endogenous parkin down-regulates p53 pathway both *in vitro* and *in vivo*.

We have examined whether FPD-associated parkin mutations could impair the protective phenotype elicited by wild-type parkin. In order to correlate a putative loss of parkin function to the abrogation of its ligase activity, we examined the influence of various familial mutations known to either abolish (C418R and C441R) or preserve (K161N and R256C) this catalytic activity. We have transiently transfected wild-type parkin or its mutants in SH-SY5Y cells and measured caspase-3 activity. With respect to PD pathology, it is first interesting to note that wild-type parkin protects the dopaminergic neuroblastoma cell line SH-SY5Y from the caspase-3 activation triggered by 6OHDA (Fig.3a), a natural dopaminergic toxin frequently used to trigger PD-like phenotypes *in vivo* [9]. Thus, wild-type parkin significantly decreases (57%) caspase-3 activity ($n=6$, $p<0.05$, Fig.3a), p53 expression (Fig.3b) and p53 promoter activity ($n=6$, $p<0.05$, Fig.3c) in 6OHDA-treated SH-SY5Y cells. Interestingly, both ligase-active and ligase-dead mutants were unable to affect 6OHDA-stimulated caspase-3 activity (Fig.3a) and increased both p53 expression (Fig.3b) and promoter transactivation (Fig.3c). It should be noted that another mutation (R42P) also abolished parkin-induced reduction of p53 in lentiviral-infected primary cultured neurons (data not shown).

We have examined if mutated parkin could rescue parkin's ability to control p53 after transfection in parkin-deficient fibroblasts. Interestingly, over-expression of C418R-parkin (Fig.3d) and other mutations (Fig.Suppl.1) all abolish the parkin-associated reduction of p53 promoter transactivation (Fig.3e) and do not affect p53 mRNA levels (Fig.3f). Overall, this set of data clearly establishes that parkin-associated down-regulation of p53 transcription is abolished by FPD mutations independently of its ubiquitin ligase activity and agrees well with

our experiments failing to demonstrate a parkin-mediated ubiquitination of p53 (data not shown).

To date, only six human brain samples are available worldwide to examine whether PD-affected patients carrying a parkin mutation display alteration in their endogenous p53 level. We had the opportunity to obtain two brain samples carrying either a point mutation or an exon deletion (see methods). Although the number of pathological samples was small, we observed a reproducible and consistent increase in p53-like immunoreactivity in FPD brains (454% of control brains, n=2, Fig.3g). This fully supports the above data and strongly suggests that the influence of parkin mutations on cellular p53 pathway could indeed reflect alterations occurring in pathological brains.

One of the main cell survival molecular pathways involves PI3-kinase-mediated phosphorylation of Akt/PKB [10]. Several studies consistently documented a molecular cascade linking Akt and NFκB ultimately leading to p53 inhibition and cell survival [11]. It was therefore of interest to examine whether the selective Akt inhibitor LY294002 could prevent parkin-associated reduction of p53 pathway. Our data indicate that LY294002 did not modulate parkin-mediated reduction of 6OHDA-stimulated caspase-3 activity (Fig.Suppl.2), suggesting that the control of p53 by parkin did occur mainly at a transcriptional level.

We have delineated the p53 domain with which parkin could functionally interact by means of deletion analysis of the 5' p53 promoter region. Fig.4a,b clearly shows that parkin-induced reduction of luciferase activity was abolished when the -312 to -196 was deleted (compare p53-4 and p53-5 constructs). This led us to examine whether parkin could physically interact with this p53 promoter region. We have designed 3 probes covering the –312 to –130 promoter sequence of p53 (Fig.4c). Our data shows that parkin only interacts with the -312 to -243 promoter region covered by Pp53-2 probe (Fig.4d, left panel) while parkin did not interact with the Pp53-1 and Pp53-3 probes (Fig.4d). Importantly, parkin-Pp53-

2 interaction was observed for both endogenous and over-expressed parkin in HEK human 293 cells (Fig.4d, right panel) and, as expected, increased labelling of the parkin-Pp53-2 complex was observed in cells over-expressing parkin. The specificity of the interaction was further supported by supershift experiments that indicate a down-regulation of the parkin-Pp53 complex in the presence of a specific antibody directed towards parkin as well as by the full displacement of the parkin-Pp53-2 labelling by a 20-fold excess of cold specific (cs) Pp53-2 probe (Fig.4d, right panel).

Two distinct set of data indirectly suggested that parkin could potentially act as a transcription factor. First, parkin could harbour a nuclear localization [4,5,12] and second, parkin contains an IBR domain that often predicts for DNA binding properties [6]. That the putative transcriptional targets of parkin could concern proteins involved in the control of cell death was suggested by the fact that over-expressed parkin was shown to antagonize ceramide-induced up-regulation of various genes including CHK, EIF4EBP1, GADD45A and PTPN-5[7] suggesting a repressing role of parkin in the control of apoptosis-associated genes. Our study clearly establishes a direct link between parkin and the tumor suppressor p53 and identifies this oncogene as the first parkin transcriptional target. It is interesting to note that several ubiquitin ligases have been implicated in the post-translational control of p53 [13-16]. Amongst them, MDM2, the major regulator of p53 binds to p53 and triggers 26S proteasome-mediated degradation and functional inactivation of p53[17]. Furthermore, MDM2 indirectly controls the transcription of p53 via the interaction with Nedd8 [18].However, our data clearly establish that parkin-mediated control of p53 remains independent of its ubiquitin-ligase activity.

Of most interest, all parkin mutations associated with familial cases of PD examined in this study abolish parkin-mediated control of the p53 pathway. This is particularly interesting when considering that parkin mutations account for the bulk of familial recessive cases of PD and that experimental models underscores p53 as a major contributor of PD-associated cell

death killing dopaminergic neurons in this pathology [19,20]. Although human brain samples were difficult to obtain, our set of anatomical pieces show confirmed that p53 was abnormally high in parkin-associated familial PD brains.

The above data could also be of potential importance in cancer. Thus, several epidemiological studies have shown a negative relationship between PD and cancer [21-23]. In addition, parkin expression is altered in several types of cancer [24] and was identified as a candidate tumor suppressor gene on chromosome 6q25-q27 [25]. Therefore, mutations on parkin levels likely contribute to the molecular dysfunctions taking place in Parkinson's disease where p53-associated cell death is exacerbated, but as well, one could speculate that parkin levels could be implicated in cancers by modulating p53 transcription.

Methods

Cell systems

$Pk^{+/+}$, $Pk^{-/-}$, $p19^{Arf-1-/-}$ and $p19^{Arf-1-/-}p53^{-/-}$ fibroblasts were obtained and cultured as described [8,26]. TSM1 neurons expressing empty vector or wild-type HA-tagged parkin were obtained after the transfection (2µg of each cDNA) by means of lipofectamine according to manufacturer's conditions (Invitrogen). HEK293 cells, TSM1 and SHSY5Y cells were grown in 5% CO_2 in DMEM supplemented with 10% fetal calf serum containing penicillin (100U/ml) and streptomycin (50µg/ml).

Caspase-3 activity Measurements

Cells were grown in 6-well plates and incubated without or with staurosporine (1µM) or of 6OHDA (0.2mM) for 2-4 and 8 hours respectively. When indicated, cells were pre-treated or not for 30 min with LY294002 (10µM) prior to 6OHDA treatment. Caspase-3-like

enzymatic activity was measured fluorimetrically by means of a microtiter plate reader (Labsystems, Fisher Bioblock Scientific) as extensively detailed [27].

Western blot analyses

Proteins (50µg) were separated on 12% SDS-PAGE gels and wet transferred to Hybond-C (Amersham Life Science) membranes. Immunoblotting was performed by means of mouse monoclonal anti-p53 antibodies (Santa Cruz, Biotechnology), mouse monoclonal anti-HA antibodies (Covance) or mouse monoclonal anti-actin antibodies (Sigma). Immunological complexes were revealed with anti-mouse IgG coupled to peroxidase (Immunotech) antibodies, followed by electrochemoluminescence revelation (Amersham Pharmacia Biotech).

p53 activity and promoter transactivation

The p53 transcriptional activity and promoter transactivation were measured as previously described [27]. 5' deletion p53 promoter constructs have been described previously [28].

Human and murine brain tissues

Both sample groups correspond to striatum (nucleus caudate/putamen). The "England" group includes one control brain and one FPD brain (female**,** 83 years**,** heterozygous missense mutation Arg275Trp in exon 7). The "Japan" group includes three control brains without history of dementia or other neurological diseases, and one FPD brain (male, 70 years, homozygous exon 4 deletion)[29], respectively. Parkin knockout murine brains have been recently described[30].

RT-PCR analysis of mouse p53 mRNAs

Total RNA from cells was extracted using the RNeasy kit following the instructions of the manufacturer (Qiagen), reverse transcribed (AMV-transcriptase, Promega) then submitted to real time PCR as described [27].

p53 Electrophoretic mobility shift Assay (EMSA)

EMSA was performed using a commercial DNA-binding-protein detection system (Promega). In brief, three couples of oligonucleotides (1F-1R; 2F-2R and 3F-3R, see below) covering distinct regions of the human p53 promoter were synthesized by Eurogentec, annealed and end-labelled using [^{32}P]ATP (6000Ci /mmol, ICN Biomedicals). For the preparation of nuclear extracts, HEK293 cells were cultured in 100mm diameter dishes and transiently transfected with either empty or HA-tagged parkin cDNA vectors (12μg), by means of lipofectamine. Forty-eight after transfection, cells were recovered and nuclear extracts were prepared according the Current Protocols in Molecular Biology. For the preparation of nuclear extracts from MEF cells, cells were cultured in 100 mm diameter dishes till 80% confluence was reached and then extracted according to protocol depicted above. Binding reactions containing nuclear extracts (10μg) were performed at 37°C using the p53 probes according to the manufacturer's conditions. The specificity of the above-described reactions was verified by a supershift assay where a pre-incubation (37°C, 10min) of nuclear extracts with either anti-HA/MAB5512 (specific) or anti-V5 (non-specific) antibodies was performed before the addition of the labelled probe and re-incubation at 37°C for 20 min. Then, protein-DNA complexes were resolved by electrophoresis (3 hours at 300V) on native polyacrylamide gels (7%) in Tris 5mM, pH8.3 buffer containing glycine (38 mM). Gels were dried and autoradiographed on a BAS-1500 phosphorimager (Fujifilm).

p53-1F: 5'GCCAGGAGCCTCGCAGGGGTTGATGGGATTGGGGTTTTCCCCTC

CCAT GTGCTCAAGACTGGCGC3',

p53-1R: 5'GCGCCAGTCTTGAGCACATGGGAGGGGAAAACCCCAATCCCATC

AACCCCTGCGAGGCTCCTGGC3',

p53-2F: 5'GGCACCAGGTCGGCGAGAATCCTGACTCTGCACCCTCCTCCCCAAC

TCCATTTCCTTTGCTTCCTCCGGC3',

p53-2R: 5'GCCGGAGGAAGCAAAGGAAATGGAGTTGGGGAGGAGGGTGCAG

AGTCAGGATTCTCGCCGACCTGGTGCC3',

p53-3F: 5'TTCCTCCGGCAGGCGGATTACTTGCCCTTACTTGTCATGGCGACTG

TCGACCTTTGTGCCAGGAGCCTCG3',

p53-3R: 5'CGAGGCTCCTGGCACAAAGCTGGACAGTCGCCATGACAAGTAAGG

GCAAGAATCCGCCTGCCGGAGGAA3'

Parkin lentivirus generation and primary cultures of neurons

The open-reading frame of human parkin fused to eGFP was inserted into the cFUGW lentiviral expression plasmid (a kind gift of Dr David Baltimore, California. Institute of Technology). Lentivirus were prepared as previously described[31]. Packaging constructs pLP1, pLP2 and pVSV-G (Invitrogen) were substituted into the production protocol. Titer of concentrated virus was directly estimated on primary neuronal cultures through visualization of eGFP four days post-infection.

Primary cortical neuron cultures were prepared from gestational day 15-16 fetal CD-1 mice (Charles Rivers). Cortices were dissected and the cells dissociated by trituration in modified Eagle's medium (MEM), horse serum (20%), glucose (25mM) and L-glutamine (2mM) following a 15 min digestion in TrypLE (Invitrogen). The cells were plated on 6-well plates coated with poly-L-ornithine and were maintained in MEM, horse serum (10%), glucose (25mM), and L-glutamine (2mM) at 37°C in a 7% CO_2 humidified incubator. The

glial cell growth was inhibited by addition of 5-Fluoro-2'-deoxyuridine (5F2DU, Sigma, 30μM) to the culture medium on DI 4. The growth medium was refreshed once every third day. At DIV6, concentrated lentivirus were directly added at a multiplicity of infection of 0.5, 1 or 2 as determined through exposure in control cultures and corresponding visualization of eGFP positive cells. At DIV10, cells were harvested into ice-cold PBS containing 1% Triton-X-100 and complete protease cocktail inhibitors (Roche).

Statistical Analysis

Statistical analysis was performed with PRISM software (GraphPad Software, San Diego, CA) by using either the t-test Student or Newmann-Keuls multiple comparison tests for one-way analysis of variance.

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Competing interests statement The authors declare that they have no competing interests.

Legends

Figure 1: Protective effect of parkin is associated to a modulation of p53 in TSM1 neuronal cell line.

Panel a: Analysis of parkin-, p53- and actin-like immunoreactivity in stably transfected TSM1 cells over-expressing either HA-tagged wild-type parkin (Pk) or empty vector (Ct) (numbers indicate distinct clones). **Panel b:** Caspase-3 activity in TSM1 neurons over-expressing empty vector (mock) and wild-type parkin (Wt-Pk) after treatment with staurosporine (STS, 1µM, 2hours) or 6-hydroxydopamine (6OHDA, 0.2 mM, 8 hours). Bars represent the means ± SEM of 5 independent experiments performed in duplicates.* $p<0.01$ (compare Wt-Pk and Mock).

Panel c: Analysis of p53 expression (p53), activity (PG13), promoter transactivation (Pp53) and mRNA levels (mRNA) in Mock- and Wt-Pk-transfected cells (clone Pk15, see a). Values are expressed as percent of mock-transfected cells (taken as 100) and are the means ± SEM of 4-5 independent experiments performed in duplicates. *$p<0.01$, **$p<0.001$, *** $p<0.0001$.

Panel d: p53 expression in lentiviral-infected primary cultured neurons over-expressing parkin. Primary cultured neurons were infected with 0.5, 1 and 2 MOI of Wt-Pk-lentiviral vector and then assayed for parkin and p53 expression four days after infection as described in the Methods**.**

Panel e and f: $p19^{arf-/-}$ and $p19^{arf-/-/p53-/-}$ fibroblasts were transiently transfected with empty pcDNA3 or parkin cDNA. Twenty-four hours after transfection, cells were treated for 8 hours with 6OHDA (0.2mM) then expression of parkin (e) and caspase-3 activity (f) were monitored as described in the Methods. **p<0.001. ns, not significant.

Figure 2: p53 pathway is up-regulated in parkin-deficient fibroblasts and mice brains.

Panel a: Caspase-3 activity in 6OHDA-treated (6OHDA, 0.2 mM, 8 hours) wild-type (Pk^+) and parkin-deficient (Pk^-) fibroblasts. Bars represent the means ± SEM of 3 independent experiments performed in triplicate. *p<0.05; **p<0.01).

Panel b: p53- and actin expressions in Pk^+ and Pk^- fibroblasts. Bars represent densitometric analysis and are the means ± SEM of 3 independent experiments performed in duplicate (**p<0.01).

Panel c: Analysis of p53 activity (PG13), promoter transactivation (Pp53) and mRNA levels (mRNA) in Pk^+ and Pk^- fibroblasts. Bars represent the means ± SEM of 3 independent experiments performed in duplicates (*p<0.05; **p<0.01).

Panel d: p53 and actin immunoreactivities in brain homogenates derived from Pk^+ and Pk^- mice. Bars represent densitometric analysis and are the means ± SEM of 4 brains analysed in duplicates. *p<0.05.

Panel e: p53 and parkin-like immunoreactivities in parkin-deficient fibroblasts transiently transfected with empty vector (Ct) or wild-type parkin (Wt-Pk) were measured as described in the Methods.

Panels f and g: promoter transactivation (f) and mRNA levels (g) in parkin-deficient cells transiently transfected with empty vector (Ct) or wild type parkin (Wt-Pk). Values are expressed as percent of mock-transfected parkin-deficient fibroblasts (controls taken as 100)

and are the means ± SEM of 3 independent experiments performed in triplicate. *p<0.05,**p<0.01,***p<0.001. ns, not statistically significant.

Figure 3: Familial Parkinson's disease associated mutations abolish parkin ability to control p53 and do not rescue parkin function in parkin-deficient fibroblasts.

Panels a-c: caspase-3 activity (a), p53 expression (b) and p53 promoter transactivation (c) in SH-SY5Y cells transiently transfected with empty vector (Ct), wild type parkin (Wt-Pk) and the indicated mutated parkin constructs. In panel b, parkin- and actin-like immunoreactivities are shown to control of transfection efficiency and protein loading. Values are expressed as percent of mock-transfected SH-SY5Y cells (controls taken as 100) and are the means ± SEM of 3 independent experiments performed in triplicates. *p<0.05, **p<0.01, ***p<0.001. Ns, not statistically significant.

Panel d: p53 and parkin-like immunoreactivities in parkin-deficient fibroblasts transiently transfected with empty vector (Ct) or C418R-parkin (mPk) cDNA were measured as described in the Methods.

Panels e and f: promoter transactivation (e) and mRNA levels (f) in parkin-deficient cells transiently transfected with empty vector (Ct) or C418R-parkin. Values are expressed as percent of mock-transfected parkin-deficient fibroblasts (controls taken as 100) and are the means ± SEM of 3 independent experiments performed in triplicates. ns, not statistically significant.

Panel g: Parkin mutations increase p53 in Parkinson's disease-affected human brains.

p53 expression and densitometric analyses in control (Ct) and pathological (FPD, Familial Parkinson's Disease) human brains (Group 1 (G1) represents the "England" samples and group 2 (G2) the "Japan" samples, see Methods).

Figure 4: Deletion analysis of p53 promoter transactivation by parkin and physical interaction between parkin and p53 promoter.

Panel a: Representation of the 5' sequential deletion constructs (p53-n) of p53 promoter region.

Panel b: p53 promoter transactivation in SH-SY5Y cells. The indicated p53 promoter-luciferase constructs were co-transfected with the β-galactosidase reporter gene and either empty cDNA or wild-type parkin (WtPk) cDNA. Bars represent the means ± SEM of 3 independent experiments performed in duplicates.* $p<0.05$; **$p<0.001$.

Panel c: Schematic representation of the human p53 promoter regions covered by the three p53 probes (Pp53-1, Pp53-2 and Pp53-3).

Panel d: (left) Gel retardation obtained when a nuclear preparation of HEK293 cells over-expressing Wt-Pk is incubated with the indicated γ-ATP ^{32}P-labelled p53 probe in absence (-) or in the presence (+) of an excess of cold p53 probe (cs= cold specific). Note that only Pp53-2 reveals a specific and displaceable Pk-p53 complex. **Panel d** (right, lanes 1,2) illustrates the gel retardation obtained when nuclear preparations of HEK293 cells transiently transfected with empty vector (Ct) or HA-tagged Wt-Pk (Pk) are incubated with labelled Pp53-2 probe; lane 3 represents lane 2 in presence of an excess of cold specific Pp53-2 probe and lanes 4 and 5 represent lane 2 in presence of non specific (ns, anti-V5) or specific (s, anti-HA) antibodies (Abs). Note that the label of the Pk-p53 complex is abolished only in the presence of specific cold probe and specific antibody.

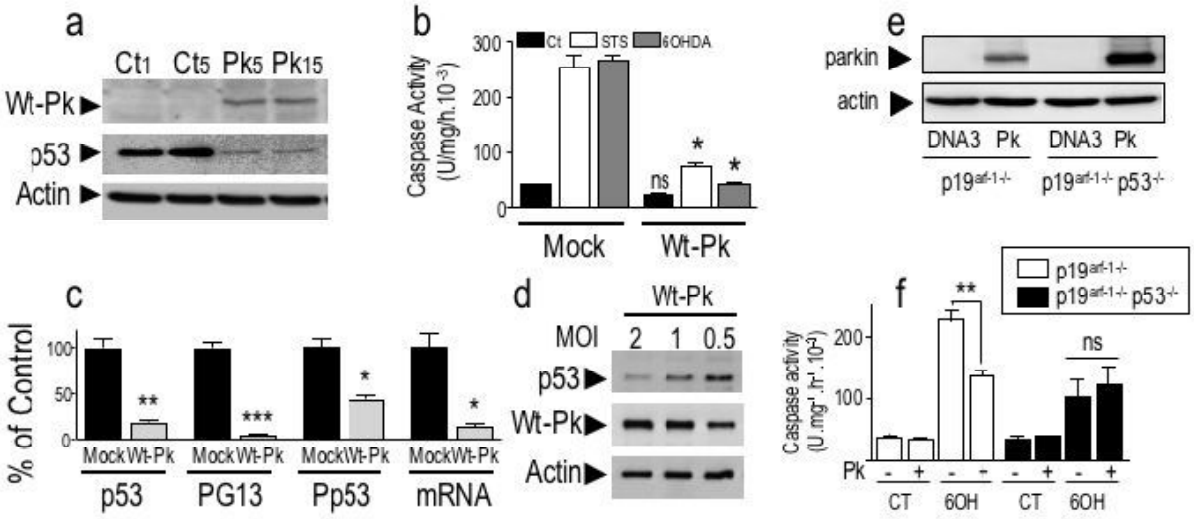

Fig.1 Alves da Costa et al.

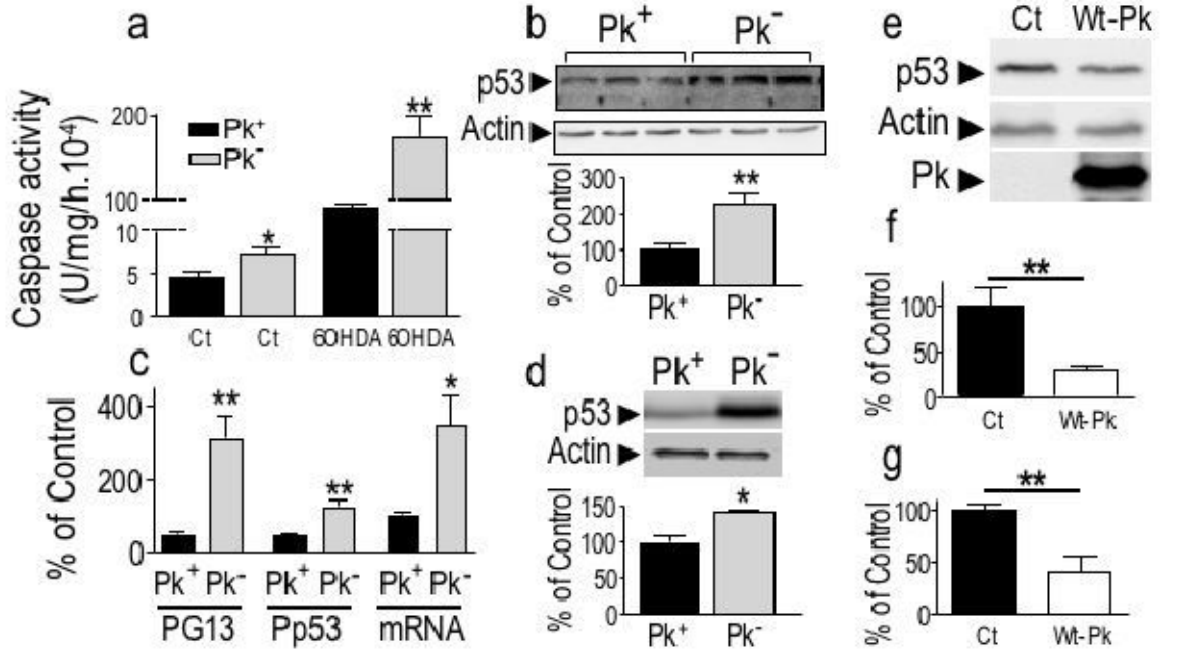

Fig.2 Alves da Costa et al.

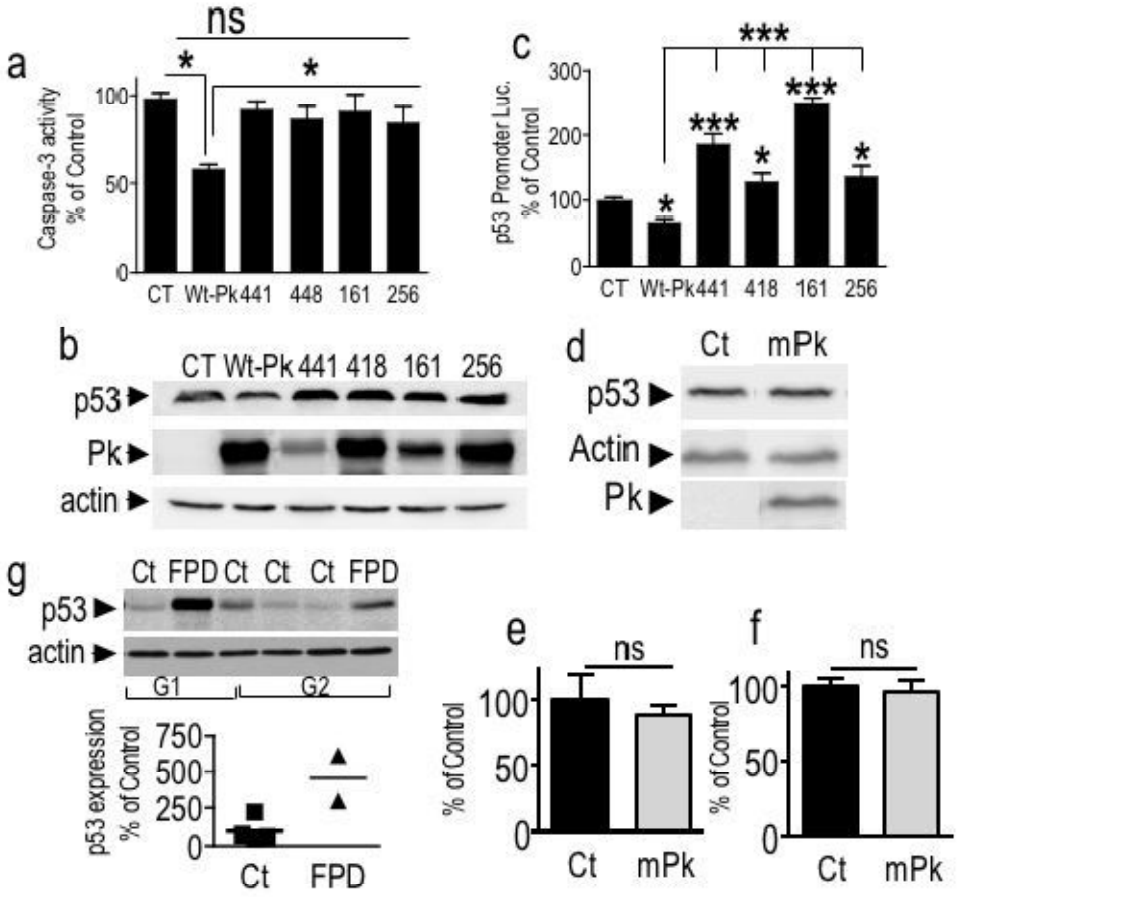

Fig.3 Alves da Costa et al.

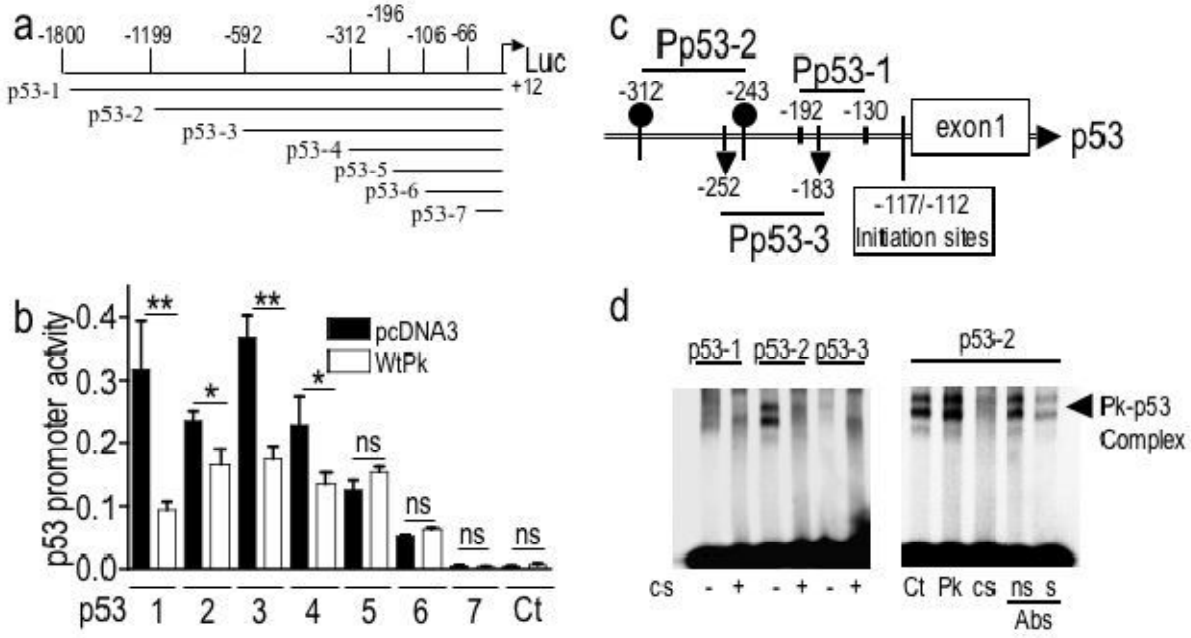

Fig.4 Alves da Costa et al.

B.IV) La Parkine régule DJ-1 par l'intermédiaire de p53 (Article 4)

Article 4: "Ubiquitin ligase independent and p53-mediated modulation of DJ-1 by parkin implication in Parkinson's disease." En préparation

Récemment, une étude du Dr Moore et collaborateurs a montré un lien entre la parkine et DJ-1 (Moore et al., 2005b). Cette étude montre que contrairement à ce que l'on attendait la parkine régule positivement DJ-1 et ne favorise pas sa dégradation. Suite au précédent projet montrant une régulation de p53 par la parkine, nous nous sommes intéressés à une possible régulation de DJ-1 par la parkine via p53.

DJ-1 est régulé positivement par la parkine :

Dans les cellules TSM1 surexprimant stablement la parkine sauvage (PK), tout comme dans des SH-SY5Y exprimant transitoirement la PK l'activité du promoteur de DJ, son taux d'ARNm, et son expression sont augmentés. Inversement, dans des cellules invalidées pour la parkine, on observe une diminution drastique de l'expression et de l'activité du promoteur de DJ-1. Nous avons donc déterminé que ce mécanisme n'est pas spécifique d'un type cellulaire. Afin de savoir si la capacité de régulation de la parkine passe par son activité d'ubiquitine ligase, nous avons transfecté dans des SH-SY5Y, les ADNc de deux mutants pathogènes de la parkine. Les mutations présentent sur la parkine préservent ou non son activité d'ubiquitine ligase. Ces deux mutations ont un effet similaire sur DJ-1. Les protéines mutées perdent la capacité de moduler DJ-1. Cette nouvelle fonction de régulation de la parkine est donc indépendante de sa fonction dans le système ubiquitine-protéasome, tout comme sa capacité à moduler p53 (Figure 1 et 2).

p53 régule négativement DJ-1 :

La parkine régule à la fois DJ-1 et p53. Nous nous sommes donc intéressés à la capacité de p53 de réguler DJ-1. De façon intéressante, l'invalidation de p53 dans des fibroblastes induit une augmentation de l'activité du promoteur, du taux d'ARNm et de l'expression de p53. Cette augmentation est inversée par la complémentation de ces cellules avec l'ADNc de p53, jusqu'à atteindre les niveaux observés dans les cellules contrôle (Figure 3).

La régulation de DJ-1 par la parkine est dépendante de p53 :

Jusqu'à présent nous avons montré que la parkine régule positivement DJ-1 alors que p53 régule DJ-1 négativement. Nous avons précédemment établit que la parkine régule négativement p53. Nous avons donc émis l'hypothèse que la parkine régule DJ-1 en modulant p53. Afin de vérifier cela nous avons surexprimé la parkine dans des cellules contrôles et des cellules invalidées pour p53. Dans les cellules invalidées pour p53 la parkine n'est plus capable de réguler DJ-1, capacité qu'elle récupère lorsque l'on complémente les cellules avec l'ADNc de p53 (Figure 4).

Conclusion/ Discussion.

Ces travaux ont montré que la parkine exerce une fonction de régulation sur DJ-1 par sa capacité de moduler à un niveau transcriptionnel p53. Cette aptitude est indépendante de son activité ubiquitine-ligase et est totalement dépendante de p53 (Figure 41). Les travaux à venir permettront de déterminer si p53 est capable de réguler DJ-1 à un niveau transcriptionnel en se fixant directement sur son promoteur. Par mutagenèse nous pourrions muter différents sites putatifs de fixation de p53 sur le promoteur de DJ-1 afin de discriminer le véritable site des autres. A l'aide de la technique de Chip nous comptons également déterminer si le site que nous aurons identifié sera effectivement un site de fixation de p53.

Article 4

Giaime E., Sunyach C., Druon C., Corti O., Brice A., Heutink P., Dawson T., Ariga H., Checler F., and Alves da Costa C.

"Ubiquitin ligase independent and p53-mediated modulation of DJ-1 by parkin implication in Parkinson's disease"

En préparation

DJ-1 protein stabilization by parkin is mediated by p53: insight to genetic crosstalk in Parkinson's disease

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Abstract

DJ-1 and parkin are two multi-functional proteins linked to autosomal recessive early-onset Parkinson's disease (PD). Parkin is an-ubiquitin-ligase that has been shown to functionally interact with DJ-1. Interestingly, instead of helping out its ubiquitinylation and proteosomal degradation, it leads to DJ-1 protein stabilization. We have studied here the mechanism by which parkin upregulates the cellular levels of DJ-1. We demonstrate that overexpression of parkin in TSM1 neurons-like and dopaminergic SH-SY5Y human neuroblastoma cells upregulate expression, mRNA levels and promoter transactivation of DJ-1. We show that this effect is not dependent of parkin ubiquitin-ligase activity since both ubiquitin and non-ubiquitin-ligase parkin mutants abolish DJ-1 stabilization in SH-SY5Y cells. Of most interest, we show a decrease of DJ-1-like immunoreactivity in fibroblasts invalidated for *parkin* gene indicating that endogenous parkin may control DJ-1 protein levels. In addition, we have established that DJ-1 is a transcriptional target of p53. Thus, overexpression and depletion of p53 lead to opposite control of DJ-1 protein and mRNA levels. Recently, we have established that parkin is responsible for the transrepression of the p53 gene. These data led us to investigate if the up-regulation of DJ-1 by parkin was not mediated by p53. We show here that the effect of parkin over DJ-1 is fully dependent of p53 In conclusion, our data suggest that parkin controls DJ-1 levels through p53. This new data give an important insight into the mechanism by which DJ-1 and parkin regulate each other in PD pathology.

Introduction

Parkinson's disease (PD) is a second most frequent neurodegenerative disorder after Alzheimer's disease. It is characterized by a severe loss of dopaminergic neurons of substantia nigra and by the presence of intra-cytoplasmic inclusions named Lewy bodies (LB). This syndrome is mainly sporadic but a thin percent of the cases are linked to a genetic origin and may be either associated with an autosomal dominant or recessive mode of transmission. The latter forms of the disease are usually associated to an early onset and are linked to mutation in the genes of *parkin*, *Pink1* and *DJ-1*.

DJ-1 is implicated in approximately 2% of familial forms of PD (1,2). DJ-1 is a ubiquitously expressed and highly conserved protein that is present as a homodimeric complex in the cytoplasm (3). To date, little is known about the physiological function of DJ-1 and the mechanisms by which DJ-1 mutations lead to PD pathology. Several lines of evidence seem to indicate that DJ-1 could be a chaperone protein (4), and that it harbours antioxidant properties (5). Several studies have demonstrated that DJ-1 can regulate the transcription of different genes like *PTEN*, *PSF* or *SOD3* (6-8), but little is known about the regulation of DJ-1 itself. Of most interest DJ-1 has been shown to functionally interact with another important familial associated gene, *parkin*. (9). Parkin is an ubiquitin-ligase that has been shown to interact and stabilize wild-type and mutated DJ-1 (9). Thus, instead of helping out the ubiquitinylation and proteosomal degradation of DJ-1 protein parkin lead to DJ-1 stabilization. This unexpected result led us to investigate the mechanism by which by parkin regulates DJ-1 in different cell systems. We have established that DJ-1 is up-regulated by endogenous and overexpressed parkin in neuronal and dopaminergic cells and that pathogenic ubiquitin and non-ubiquitin associated mutations of the *parkin* gene abolish this control. We have demonstrated that DJ-1 is a transcriptional target of p53 and that the regulation of DJ-1 protein levels by parkin is carried out by the intermediate of p53. Finally, our data give insight to the mechanistic pathway by which two major PD genes regulate each other and highlight the importance of the crosstalk between PD-associated genes to PD pathology.

Experimental procedures

Cell systems and Transfections

Telencephalon Specific Mouse 1 (TSM1), Mouse Embryonic fibroblasts (MEF) and SH-SY5Y human neuroblastoma cells were cultured in 5% CO_2, in DMEM supplemented with 10% fetal calf serum containing penicillin (100U/ml) and streptomycin (50µg/ml). TSM1 neurons expressing empty vector or wild type HA-tagged parkin were obtained as described (Alves da Costa). SH-SY5Y, $p19^{Arf-/-}$ and $p19^{Arf-/-}$p53-/- fibroblasts (10) were carried by means of either lipofectamine reagent or Nucleofactor™ kit according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France, and Amaxa Biosystems, Koeln, Germany) as previously described (11).

Western Blot Analysis

Cells were homogenized in lysis buffer 10mM Tris-HCl pH7.5, containing 150mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate and 5mM EDTA and resolved on 12% SDS-PAGE gel and wet transferred to Hybond-C (Amersham Life science, GE Healthcare, UK Buckinghamshire, England) membranes. Immunoblotting was performed by means the adequate antibodies: rabbit polyclonal anti-DJ-1 antibodies (Abcam, Paris, France), mouse monoclonal anti-HA antibodies (Covance, New Jersey, USA), mouse monoclonal anti-p53 antibodies (Santa-Cruz Biotechnology, Santa-Cruz, CA,USA) or mouse monoclonal anti-actin antibodies (Sigma, St. Quentin-Fallavier, France). Protein immunoreactivities were revealed with either an anti-rabbit peroxidase or an anti-mouse peroxidase (Jackson Immunoresearch, Cambridgeshire, UK) by electrochemoluminescence method (Roche Diagnostics S.A.S., Meylan, France). Chemiluminescence was recorded using a luminescence image analyzer LAS-3000 (Raytest,Corbevoie, France), and quantification of images were performed using the Aida Image Analyzer software.

Real-time quantitative PCR

Total RNA from cells was extracted using the Nucleospin RNA II kit following the instructions of manufacturer (Macherey-Nagel GmbH & Co. KG, Düren, Germany). After extraction 2µg of total RNA was reverse-transcribed using the Transcriptor Reverse Transcriptase (Roche) and oligo (dT) priming. Real-time PCR was performed in the Light Cycler 480 (Roche), using the SYBR Green detection protocol recommended by

manufacturer. Specific-gene primers were designed by means of the Primer Express software (Applied Biosystems, CA, U.S.A) and are the following: mouse DJ-1-specific primers : forward, 5'-GGAGATGCAAAAACGCAGGG-3' and reverse, 5'-TCCTCCTGGAAGAACCACCA-3'; human DJ-1-specific primers: forward, 5'-GCCTGATTCTTACAAGCCGG-3' and reverse, 5'-CAAGCGCAAACTCGAAGCT-3'. Relative expressions levels of mouse and human DJ-1 genes were normalized for RNA concentrations with mouse γ-actin and the human GAPDH genes using the following primers respectively: forward, 5'-CACCATCGGTTGTTAGTTGCC-3' and reverse, 5'-CAGGTGTCGATGCAAACGTT-3'; forward, 5'-TGGGCTACACTGAGCACCAG-3' and reverse, 5'-CAGCGTCAAAGGTGGAGGAG-3'.

DJ-1 promoter transactivation

The transcriptional activation of the human DJ-1 promoter (*hpDJ-1*) was measured after transfection of the cDNA coding for this promoter sequence in frame with luciferase (provided by Dr. Ariga, Sappor, Japan) as previously described (12). Activities were measured after co-transfection of the promoter cDNAs and β-galactosidase cDNA, in order to normalize transfection efficiencies (Promega, Madison, WI).

Statistical Analysis

Statistical analysis were performed with GraphPad Prism software (GraphPad Software, San Diego, CA) by using either the unpaired Student's test for pair wise comparison or Newmann-Keuls multiple comparison tests for one-way analysis of variance.

Results

Control of DJ-1 by overexpressed and endogenous parkin and effect of ubiquitin and non-ubiquitin parkin associated mutations.

Stable transfection of wild-type parkin (WTPK) in TSM1 neurons induce an increasing of 30 to 40% of endogenous DJ-1 expression (n=10, $p<0.05$, Fig.1A, B), mRNA level (n=11, $p<0.05$, Fig.1C) and promoter transactivation (n=4, $p<0.05$, Fig.1D). Inversely, depletion of *parkin* gene in fibroblasts leads to a drastic decrease of DJ-1 expression (Fig1E) and DJ-1 promoter transactivation (n=3, $p<0.001$, Fig.1F). Taking advantage of the human SH-SY5Y neuroblastoma cell line, which is considered as one of the most relevant dopaminergic cell models, we have first confirmed that WTPK overexpression in these cells also induce an increase to DJ-1 expression (n=10, $p<0.05$, Fig.2A, B), mRNA level (n=12, $p<0.05$, Fig.2C) and promoter transactivation (n=6, $p<0.05$, Fig.2D). Interestingly, familial Parkinson's disease-associated mutations known to abolish (C418R) or preserve (K161N) the ubiquitin-ligase activity of parkin both abolish this regulation (Fig. 2A-D, n=6-12, ns=non-significative). These data indicate that the capacity of parkin to modulate DJ-1 is independent to its ubiquitin-ligase activity and suggests a transcriptional regulation of DJ-1 by parkin.

DJ-1 transcriptional control of p53

Several works have demonstrated a link between p53 and DJ-1 mediated cell death control (13,14). Thus, we have shown that the caspase-6 generated C-terminal fragment of DJ-1 regulates p53 at a transcriptional level (15). Based on the well recognized transcription factor properties of p53 and its major role to PD pathology we decided to investigate if *DJ-1* was a p53 gene target. We took advantage of two cell models in which either $p19^{Arf}$ or $p19^{Arf}$ and *p53* genes had been invalidated. The depletion of $p19^{Arf}$ allows the analysis of the function of p53 in cell death but not cell cycle control (16). When we compare the $p19^{Arf-/-}$ (white bars) and $p19^{Arf-/-}$*p53-/-* (black bars) we can observe that depletion of p53 induce an increase of DJ-1 expression (n=5-7, $p<0.001$, Fig.3A, B), mRNA level (n=4, $p<0.001$, Fig.3C) and promoter transactivation (n=5, $p<0.001$, Fig.3D). Complementation of $p19^{Arf-/-}$ (white bars) and $p19^{Arf-/-}$*p53-/-* (black bars) fibroblasts with the p53 cDNA (Fig. 3C) leads to a diminution of DJ-1 expression (n=5-7, $p<0.01$, $p<0.001$, Fig.3A, B), mRNA level (n=4, $p<0.01$, $p<0.001$, Fig.3C) and promoter transactivation (n=5, $p<0.05$, $p<0.001$, Fig.3D). We can also observe that between samples of $p19^{Arf-/-}$ transfected with empty vector (DNA3) and

p19$^{Arf-/-}$p53-/- complemented in p53 the levels of DJ-1 are identical indicating that the complementation with p53 fully restores DJ-1 protein, mRNA and promoter activity levels. These results clearly demonstrate that p53 down regulate DJ-1 at a transcriptional level.

The regulation of DJ-1 by parkin is dependant of the presence of p53.

Recently, we have demonstrated that parkin can negatively regulate p53 at a transcriptional level by directly interacting with its promoter (17). Thus, as shown in Fig.4A cells stably overexpressing parkin (WTPK) drastically decrease p53 expression (n=4, $p<0.001$, Fig.4A,B). The fact that p53 downregulation and parkin upregulation both produce an increase of DJ-1 levels lead us to investigate if DJ-1 stabilization by parkin was not mediated by p53. We have overexpressed WTPK in *p19$^{Arf-/-}$* (white bars) and p19$^{Arf-/-}$p53-/- (black bars) (Fig.4 C,D) and we have observed a significant increase of expression (n=10, $p<0.01$, Fig.4 C,D), mRNA level (n=11, $p<0.05$, Fig.4E) and promoter transactivation (n=4-5, $p<0.01$, Fig.4F) of DJ-1 by parkin in p19$^{Arf-/-}$, but not in cells devoid of p53. Moreover, complementation of p19$^{Arf-/-}$ and p19$^{Arf-/-}$p53-/- cells with p53 cDNA fully restores the ability of parkin to stabilize DJ-1 (Fig 4F). This set of experiments demonstrate that the capacity of parkin to regulate DJ-1 is dependant of the presence of p53.

Discussion

DJ-1 is a mitochondrial ubiquitous protein that has been associated to sporadic and genetic PD. Its function is far from been elucidated but several studies suggest that it may be implicated in multiple functions, including fertility, cell death, cancer, RNA binding, chaperoning and oxidative stress (for review see Alves da Costa). Among all these functions the most relevant to PD pathology is the ability of DJ-1 to both control oxidative stress and cell death processes (5,18-20). Interesting, a number of studies have demonstrated the implication of DJ-1 in the regulation of gene transcription. Thus, DJ-1 may transcriptionally regulate oxidative stress associated genes, like SOD (8) and key apoptotic mediators. Thus, we have shown that DJ-1 upregulation leads to a decrease of p53 mRNA levels and promoter activity (15). These data is consistent with the observation that DJ-1 regulates bax levels via the trans-repression of p53 and that the knockout of DJ-1 in the *zebrafish* induce an upregulation of p53 and bax (14). In addition to p53, DJ-1 was also shown to trans-repress another major tumor suppressor gene, *PTEN* (6). Interestingly, the regulation of PTEN by DJ-1 suggests a crosstalk of DJ-1 with another PD-associated protein, PINK-1 (for PTEN-

induced kinase-1). Indeed, the overexpression of DJ-1 enhances the steady-state levels of PINK-1 leading to a synergistic effect of these proteins in the control of cell death (21).

Interestingly, even if, several DJ-1 transcriptional target genes have been identified, little is known about the processes and mechanisms underlying its own transcriptional and post-transcriptional regulation. Recently, a study aiming to test the putative degradation of DJ-1 by parkin, has unexpectedly evidenced that parkin was able to stabilize DJ-1 expression (9). Parkin is a familial-associated Parkinson's disease protein that till date has been mainly implicated in protein degradation due to its ubiquitin-ligase activity (22). Lately, we have demonstrated that parkin harbors transcriptional activity and that it may control p53 (17). We have demonstrated that parkin can regulate the expression and mRNA levels of p53 by directly interacting with p53 promoter (17). Due to the well-recognized role of p53 in the control of gene transcription, we decided to investigate if p53 was able to control DJ-1 expression and by consequence the parkin-mediated DJ-1 up-regulation. Here we demonstrate that the overexpression of parkin in various systems including primary cultured neurons, TSM1 neurons, SH-SY5Y neuroblastoma and fibroblasts lead to up-regulation of the metabolism of DJ-1. Of most importance, the depletion of endogenous parkin triggers an opposite phenotype. We also show that p53 can negatively regulate DJ-1 at a transcriptional level by modulating its mRNA levels and promoter activity. Furthermore, we evidence that DJ-1 stabilization by parkin is dependent of p53, indicating that DJ-1 is not a parkin direct target. Of most interest different mutants of parkin known to abrogate (C418R) or preserve (K161N) its ubiquitin-ligase activity both abolish parkin ability to stabilize DJ-1, corroborating the hypothesis of a transcriptional regulation of DJ-1 by parkin via p53. Altogether, our results suggest a cascade of events in which overexpressed parkin transcriptionally downregulates p53 that in turn up-regulates DJ-1 expression. Thus, one can imagine that in PD pathology, parkin loss of function mutations would lead to an increase of p53 levels that in turn would lead to DJ-1 destabilization. Since both DJ-1 and parkin have been shown to harbor neuroprotective properties (15,17,20,23), the loss of a positive crosstalk between parkin and DJ-1 would ultimately potentiate neurodegeneration in PD.

Overall, this work gives insight into the mechanism by which DJ-1 and parkin functionally interact to control cell death in PD.

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Legends

Figure 1: Overexpression and deletion of parkin inversely modulate DJ-1 levels .

(A, E) DJ-1 and actin expression in either TSM1 cells or in MEF cells invalidated ($PK^{-/-}$) or not ($PK^{+/+)}$ for the *parkin* gene. (B) Quantification analysis of the experiences depicted in A. Bars represent the means ± SEM of 10 independent experiments performed in triplicate. (C) Analysis of DJ-1 mRNA levels in TSM1 cells monitored as described in the "Experimental Procedures". Bars represent the means ± SEM of 11 independent experiments. (D, F) DJ-1 promoter transactivation determination in TSM1 neurons or in $PK^{+/+}$ and $PK^{-/-}$ fibroblasts monitored as described in the "Experimental Procedures. Bars represent the means ± SEM of (D) 4 independent experiments performed in 6 replicates or (F) 3 independent experiments performed in 5-6 replicates. *p<0.05, **p<0.01, ***p<0.001.

Figure 2: Familial Parkinson's disease associated mutations abolish parkin ability to up-regulate DJ-1.

SH-SY5Y neuroblastoma cells were transiently transfected with the indicated cDNAs. Twenty four hours after transfection, (A) DJ-1, parkin and actin-like immunoreactivities were monitored as described in the "Experimental Procedures". (B) Bars represent densitometric analysis of DJ-1 immunoreactivity (A) and are the means ± SEM of 10 independent experiments. (C) cells were transiently transfected with the indicated cDNAs and DJ-1 mRNA levels were monitored as described in the "Experimental Procedures". Bars represent the means ± SEM of 12 independent experiments. (D) DJ-1 promoter transactivation analysis was performed after co-transfection of the indicated parkin cDNAs and the DJ-1 promoter construct as described in the "Experimental Procedures". Bars represent the means ± SEM of 6 independent experiments performed in 6 replicates. *p<0.05.

Figure 3: p53 modulates DJ- at a transcriptional level.

$p19^{Arf-/-}$ (white bars) and $p19^{Arf-/-}p53^{-/-}$ (black bars) fibroblasts were transiently transfected without or with p53 cDNA. Twenty four hours after transfection, (A) DJ-1, p53 and actin-like immunoreactivity were monitored as described in the "Experimental Procedures". (B) Bars represent densitometric analysis of DJ-1 immunoreactivity (A) and are the means ± SEM of

5-7 independent experiments performed in triplicates. (C) mRNA levels of DJ-1 monitored as described in the “Experimental Procedures”. Bars represent the means ± SEM of 4 independent experiments. (D) Analysis of DJ-1 promoter transactivation. Bars represent the means ± SEM of 3 independent experiments performed in 5 replicates. *p<0.05, **p<0.01, ***p<0.001.

Figure 4: Parkin regulates DJ-1 via p53.

(A) Expression of p53 in TSM1 cells stably transfected with empty vector (Mock) or of parkin (PK) cDNAs. Bars represent densitometric analysis and are the means ± SEM of 4 independent experiments. (B-F) $p19^{Arf-/-}$ (white bars) and $p19^{Arf-/-}p53^{-/-}$ (black bars) fibroblasts transiently transfected without (DNA3) or with parkin (PK) cDNAs. Twenty four hours after transfection (B) DJ-1, parkin and actin-like immunoreactivities were monitored as described in the “Experimental Procedures”. (C) Bars represent densitometric analysis of DJ-1 immunoreactivity and are the means ± SEM of 10 independent experiments. (D) DJ-1 mRNA levels monitored as described in the “Experimental Procedures”. Bars represent the means ± SEM of 11 independent experiments. (E) Analysis of DJ-1 promoter transactivation monitored as described in the “Experimental Procedures”. Bars represent the means ± SEM of 4-5 independent experiments performed in 6 replicates. (F) $p19^{Arf-/-}$ (white bars) and $p19^{Arf-/-}p53^{-/-}$ (black bars) fibroblasts were transiently transfected without or with parkin and p53 cDNA. Twenty four hours after transfection, DJ-1, parkin, p53 and actin-like immunoreactivities were monitored as described in the “Experimental Procedures”. *p<0.05, **p<0.01, ns, not statistically significant.

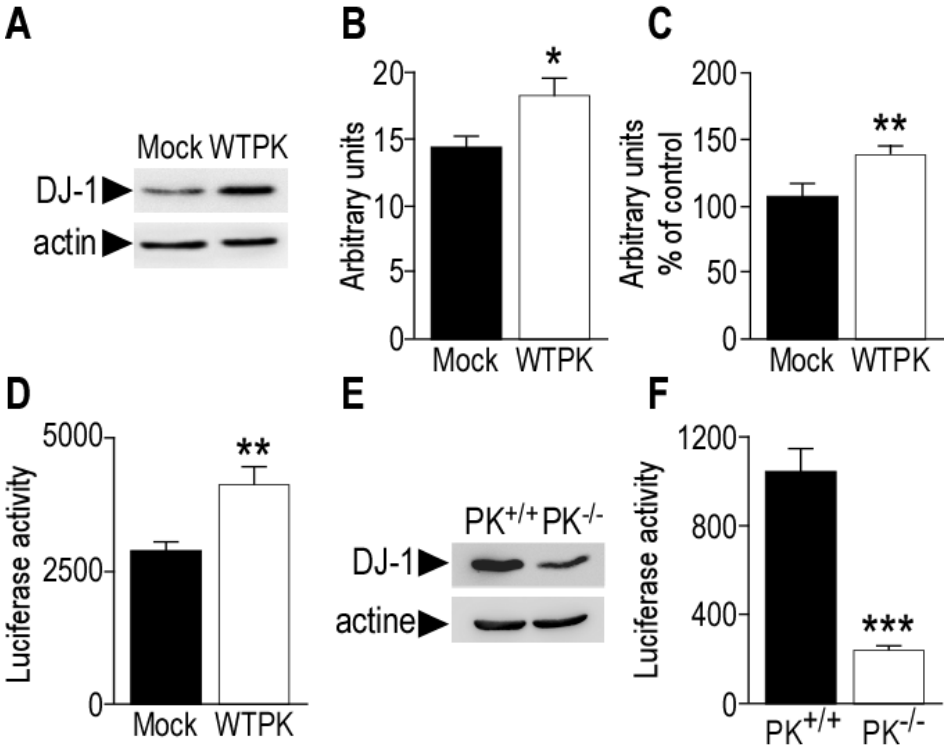

Fig.1 Giaime et al.

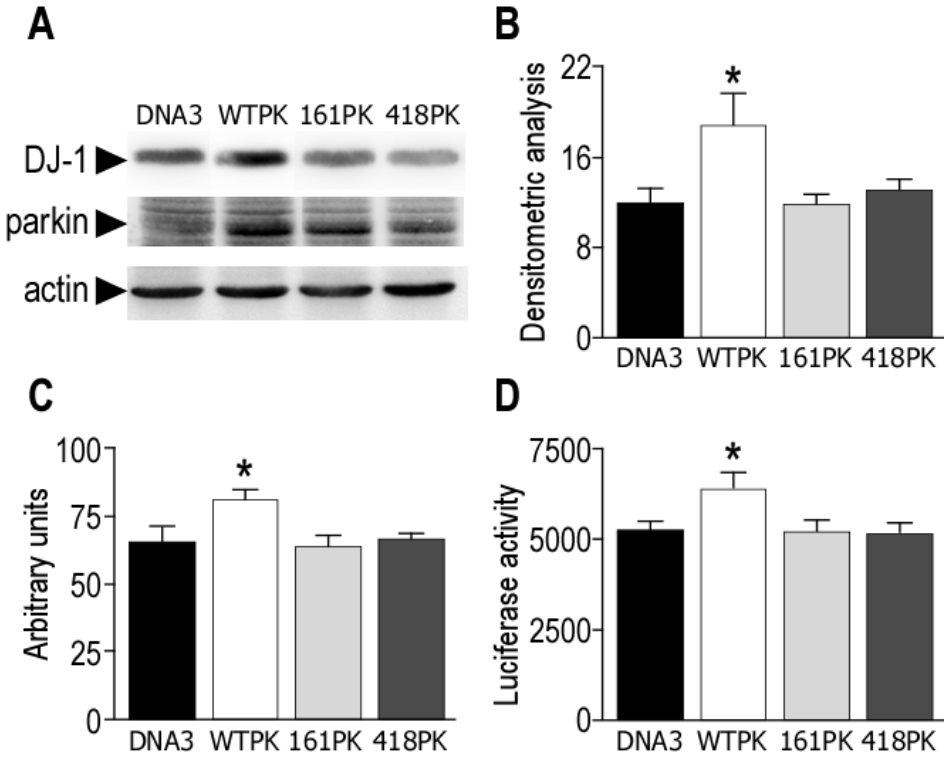

Fig. 2 Giaime et al.

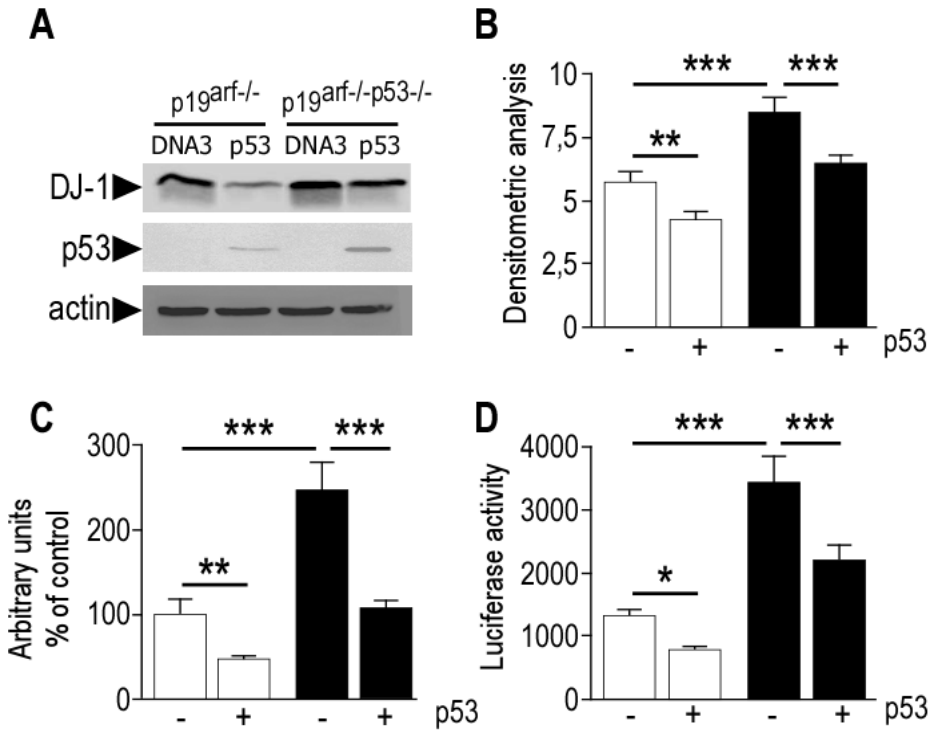

Fig.3 Giaime et al.

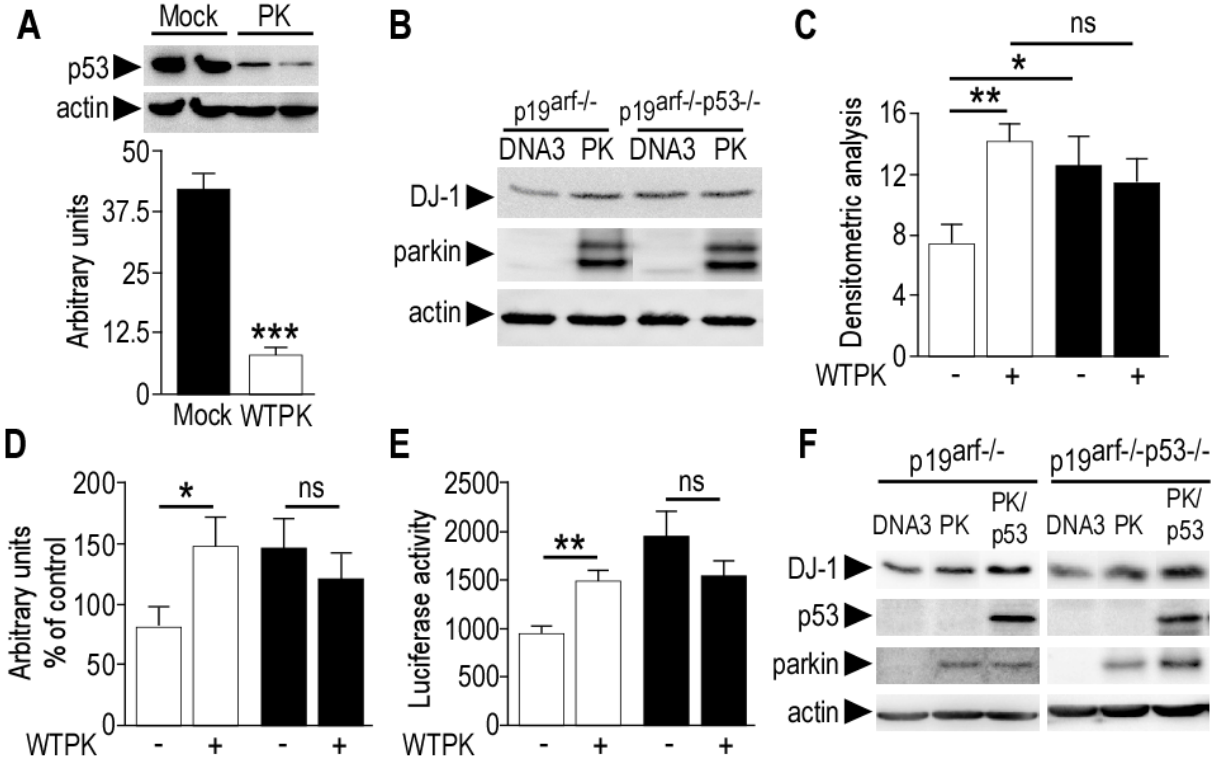

Fig. 4 Giaime et al

B.V) Autres Travaux (Article 5, 6, 7, et 8)

En dernier lieu, cette partie des résultats présente de façon succincte des travaux auxquels j'ai également collaboré.

B.V.2) Identification d'un nouveau partenaire de la Synphiline-1 (Article 5)

Article 5: "Periphilin is a novel interactor of synphilin-1, a protein implicated in Parkinson's disease". En révision.

Durant mon doctorat, j'ai également eu l'opportunité de collaborer avec l'équipe du Dr Riess sur un projet concernant l'identification et l'étude fonctionnelle d'un nouveau partenaire de la synphiline-1, la périphiline. Cette étude confirme une interaction *in vitro* et in vivo de ces 2 protéines ainsi que leur co-localisation dans des cellules et dans les corps de Lewy. Dans cette étude j'ai montré une fonction protectrice de la périphiline. Cette protéine est capable de réduire l'activité des caspases effectrices suite à un traitement à la 6-OHDA. J'ai également montré qu'il n'existe pas de synergie au niveau de la fonction protectrice entre la périphiline et la synphiline-1. Nous avons identifié une mutation au niveau du gène de la périphiline chez des patients parkinsoniens, ainsi qu'une augmentation de l'expression de cette protéine dans les régions affectées dans la maladie de Parkinson. Cependant la périphiline mutée présente toujours une fonction protectrice. Tout ceci démontre que cette nouvelle protéine pourrait avoir un rôle important dans la maladie de Parkinson.

Article 5

Soehn A.S., Franck T., Biskup S., Floss T., Trang P., Vogt Weisenhorm D.M., **Giaime E**., Cebo D., Berg D., Melle C., Strauss K.M., Rott R., Engelender S., Kalbacher H., Ott E., Tomiuk J., Von Eggeling F., Pahnke J., Meitinger T., Aho S., Krüger R., Alves da Costa C., Wurst W., Gasser T., Riess O.

"Periphilin is a novel interactor of synphilin-1, a protein implicated in Parkinson's disease".

En révision

TITLE PAGE

Title:

Periphilin is a novel interactor of synphilin-1, a protein implicated in Parkinson's disease

Running title: Periphilin in PD

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder characterized by the loss of dopaminergic neurons and the presence of intracytoplasmic inclusions (Lewy bodies). Alpha-synuclein and its interactor synphilin-1 are major components of Lewy bodies in PD patients. Rare mutations in the *α-synuclein* and *synphilin-1* genes have been implicated in the pathogenesis of PD, however, the normal function of these proteins is far from being completely elucidated. We thus searched for novel synphilin-1 interacting proteins by yeast two-hybrid screening and deciphered periphilin as new interactor. This interaction was confirmed by co-immunoprecipitation in HEK293 cells and in a mouse model transgenic for synphilin-1. We show that periphilin displays an overlapping expression pattern with synphilin-1 in HEK293 cells, subcellular fractions of rat brain lysate, and Lewy bodies in brains of PD patients. Searching for mutations in the *periphilin* gene, we detected a K69E substitution in two affected patients of a small family with autosomal dominant PD. Functional studies demonstrate that periphilin displays an antiapoptotic function in the control of cell death, as periphilin reduces basal and 6-hydroxydopamine-stimulated caspase-3 activity. Moreover, in adult mice, expression of periphilin is strongest in brain regions affected in PD. We therefore conclude that periphilin is a novel member of protein interaction networks altered in PD.

ABBREVIATIONS

5'UTR, 5' untranslated region; *6-OHDA,* 6-hydoxydopamine; aa, amino acid(s); ACE, 3-amino-9-ethylcarbazole; ADPD, autosomal dominant PD; bp, base pairs; CDS, coding sequence; co-immunoprecipitation (co-IP); DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme linked immunosorbent assay; ES cell, embryonic stem cell; ESI-MS, electrospray ionization mass spectrometry; FCS, fetal calf serum; GGTC, German Genetrap Consortium; HEK cells, human embryonic kidney cells; IDM, Interaction Discovery Mapping; KLH, keyhole limpet hemocyanin; LB, Lewy body; LSM, laser scanning microscope; MAP, multiple antigen peptide; NDS, normal donkey serum; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; PD, Parkinson's disease; PFA, paraformaldehyde; PMF, peptide mass fingerprint; RACE, 5' rapid amplification of cDNA ends; RIPA, radioimmunoprecipitation; RP-HPLC, reversed phase high performance liquid chromatography; RT, room temperature; RT-PCR, reverse transcription polymerase chain reaction; SELDI-MS, surface enhanced laser desorption ionization-mass spectrometry; TBS, Tris-Buffered Saline; TBST, Tris-Buffered Saline containing 0.1% Tween-20; TFA, trifluoroacetic acid; TH, tyrosine hydroxylase; wt, wild type; VTA, ventral tegmental area; Y2H, yeast two-hybrid

INTRODUCTION

Alpha-synuclein is a key protein in the pathogenesis of Parkinson's disease (PD) based on the identification of three missense mutations (A53T, A30P und E46K) and multiplications of the *α-synuclein* locus causing autosomal dominant PD (ADPD), reviewed in (1). In addition, α-synuclein is a major component of Lewy bodies (LB), the histological hallmark of PD. Its biological function is not well defined yet. It is a highly conserved presynaptic protein that is supposed to be involved in membrane fusion and budding of synaptic vesicles, for review see (1) and references therein. Downregulation of α-synuclein by antisense oligonucleotides caused a selective reduction of the presynaptic vesicular pool, suggesting that α-synuclein may regulate the size of specific synaptic pools, thereby modulating synaptic plasticity (2). Moreover, the central position of α-synuclein in a complex network of pathogenic factors is apparent from its numerous interactors, e.g. SIAH 1 and 2 (3), 14-3-3 proteins (4), S6' proteasomal protein (Tbp1) (5), human dopamine transporter (hDAT) (6), UCH-L1 (7), parkin (8), and mitochondrial cytochrome C oxidase (COX) (9).

The first protein which has been identified as α-synuclein-interactor is synphilin-1 (10). As a component of LB in the brains of sporadic PD patients and as substrate of the E3 ligases parkin and SIAH 1 and 2, synphilin-1 provides several links to neurodegeneration in PD, for review see (11). However, there is still not much known about the physiological functions of synphilin-1. The cytosolic 919-amino acid (aa) protein contains several motifs propagating protein-protein-interactions, namely ankyrin-like repeats, a coiled-coil domain, and an ATP/GTP binding domain (10), supporting a role of synphilin-1 as adapter molecule. Importantly, synphilin-1 displays antiapoptotic function in the control of cell death (12). The authors showed that synphilin-1 protects neuronal and human embryonic kidney (HEK293) cells from staurosporine- and 6-hydoxydopamine- (6-OHDA-) induced toxicity by reducing caspase-3 activity and poly(ADP-ribose) polymerase (PARP) cleavage and by diminishing both p53 expression and transcriptional activity. Interestingly, synphilin-1 is cleaved by caspase-3 and it is the C-terminal caspase-3-derived fragment that is responsible for the antiapoptotic phenotype of synphilin-1 (12).

We recently identified a novel R621C mutation in the *synphilin-1* gene in two sporadic PD patients that mediates toxicity in different paradigms of cellular stress *in vitro* (13). To further elucidate the role of synphilin-1 in the pathogenesis of PD, we executed a yeast two-hybrid (Y2H) screening for novel interacting proteins and identified periphilin as interactor of synphilin-1. Periphilin has been previously described as potential constituent of the keratinocyte cornified envelope (14) and as a gastric cancer-

associated isoform (15). Another isoform retards S-phase progression (16), but no report has linked periphilin to PD before. We generated periphilin gene trap mice which allowed us to study the expression in embryonic and adult mice, and we investigated whether periphilin might play a role in the pathogenesis of PD applying immunohistochemical studies on human PD brain, mutation analyses, and cytotoxicity tests.

RESULTS

Identification of periphilin as interactor of synphilin-1

Using a LexA based Y2H screen, we identified periphilin as interactor of synphilin-1. To test the interaction between synphilin-1 and a brain specific periphilin isoform, periphilin cDNA was isolated from human brain. The coding sequence (CDS) has a total length of 939 base pairs (bp) encoding 313 aa and lacks exon 1 as part of the 5' untranslated region (5'UTR) and exons 2, 4, and 12-14 due to alternative splicing (Fig. 1A). The exclusion of exon 2 deletes the first in-frame translation initiation codon; the second ATG is referred to as Met-1 in our numbering. In pairwise Y2H interaction tests, we co-transformed plasmids encoding periphilin (aa 1-313) fused to a GAL4 activation domain and fragments of synphilin-1 (aa 177-348 or 557-920) fused to a LexA domain into yeast strain L40 (Fig. 1). The first 176 aa of the synphilin-1 protein were deleted since they caused self-activation due to an acidic activation domain. No interaction was observed between the truncated 5' synphilin-1 construct and periphilin (data not shown). In contrast, co-transformation of the 3' region of synphilin-1 (aa 557-920 of wild type (wt) and R621C synphilin-1) revealed a strong β-galactosidase reporter activity indicating an interaction between periphilin and both synphilin-1 variants (Fig. 2). The K69E mutation (described in a subsequent section) in the *periphilin* gene of two PD patients did not affect the interaction (Fig. 2). Fine mapping of the interaction site proved that the carboxyterminal portion of synphilin-1 (both wt and R621C) interacts with aa 25-313 and 136-313 of periphilin but not with aa 136-287, indicating that aa 288-313 are necessary for synphilin-1-binding in yeast (Fig. 2).

To confirm the interaction with independent methods, we first applied a combination of co-immunoprecipitation (co-IP) and surface enhanced laser desorption ionization-mass spectrometry (SELDI-MS). This method was chosen, since SELDI-MS provides a very sensitive method to detect co-immunoprecipitated proteins (17), even if they are as highly insoluble as periphilin (14), which can hamper the detection of co-immunoprecipitation by Western Blotting. For this assay, lysates from HEK cells overexpressing V5-tagged periphilin and FLAG-tagged synphilin-1 were immunoprecipitated using V5 antibodies conjugated to agarose beads and non-specific antibodies coupled to Interaction Discovery Mapping (IDM) beads as negative control, respectively. The co-immunoprecipitated proteins were subjected to SELDI-MS. Comparing V5-IP and control-IP (Fig. 3A) we detected specific signals at 101.2 kD and at 39.5 kD which correspond to the calculated molecular masses of FLAG-tagged synphilin (101.5 kD) and V5-tagged periphilin (39.5 kD; www.expasy.ch). For protein identification, the eluted proteins were tryptic digested to analyze the fragment masses by mass spectrometry (Fig. 3B).

Database searches with the peptide fragments that could be assigned to synphilin-1 revealed co-immunoprecipitation of synphilin-1 with periphilin (estimated Z score 2.35).The fragments covered 28% of the synphilin-1 protein. Periphilin was also identified in the immunoprecipitates with a coverage of 41% using the same approach (Fig. 3B; Table 1).

The periphilin-synphilin interaction was further confirmed with an immuno-deplete assay with lysates from HEK293 cells overexpressing V5-tagged periphilin and FLAG-tagged synphilin-1. SELDI-MS analysis demonstrated that the signals corresponding to periphilin as well as to synphilin-1 were depleted in the supernatant after immunoprecipitation of periphilin. In a negative control using a non-specific antibody both signals were clearly detectable (Fig. 3C-D).

To validate the interaction between periphilin and synphilin-1 in an *in vivo* situation, we immunoprecipitated synphilin-1 from transgenic mice overexpressing myc-tagged human synphilin-1 (T. Franck, unpublished) and were able to show co-immunoprecipitation of endogenous periphilin protein (Fig. 4). This firmly underlines the interaction of both proteins by showing that also endogenous periphilin interacts with synphilin-1 *in vivo*.

Co-localization of periphilin and synphilin

We investigated if the two proteins share similar subcellular compartments *in vitro* and *in vivo*. Confocal laser scanning microscopy revealed endogenous periphilin predominantly in the cytoplasm of HEK293 cells (Fig. 5A). However, a weak staining is also apparent in the nucleus. Synphilin-1 also localizes to the cytoplasm (Fig. 5B), as previously described (10, 13) and therefore co-localizes with periphilin (Fig. 5C).

To further study the subcellular distribution of periphilin with an independent method, subcellular fractions were obtained by a series of differential centrifugations of adult rat brain homogenate. Similar to synphilin-1, periphilin was found to be present in a synaptosomal fraction (P2) (Fig. 5D-E). After hypotonic lysis of synaptosomes, both periphilin and synphilin-1 were present in the crude synaptic vesicle fractions LP1 and LP2 (Fig. 5D-E and Ribeiro et al., 2002). Although periphilin was not enriched in the LP2 fraction like synphilin-1, the presence of periphilin in this fraction suggests that periphilin and synphilin-1 may interact at the presynaptic terminal. Furthermore, the enrichment of periphilin in the LP1 fraction suggests that periphilin may also play a role at the postsynapse (18, 19).

Periphilin is expressed in the cortex of PD patients and healthy controls and is a component of Lewy bodies

Western blot analyses of lysates from postmortem human brains revealed no differences in the cortical periphilin expression between a PD patient and a control individual with no evidence of neurological disease (Fig. 6C). This applies both to the expression level and protein size, excluding posttranslational cleavage of the periphilin protein in this brain region.

In brains of PD patients, about 30% of the LB showed a prominent staining (Fig. 6A). Preabsorbtion of the antibody with the antigenic peptide in a molar ratio of 1:900 completely blocked the signal (Fig. 6B) indicating that it is specific for periphilin. In control sections of substantia nigra from patients without PD, faint immunoreactivity was observed primarily in neuronal cytoplasm, axons, and neurites, respectively.

Identification of a K69E substitution in two related PD patients of one family

We searched for periphilin mutations in familial PD and sequenced the *periphilin* gene in eight symptomatic members of four PD families. The *periphilin* gene maps to human chromosome 12q12 close to the former candidate region of *PARK8*. By the time we started our analyses, the *PARK8* gene had not been cloned yet. We therefore preferentially selected families with linkage to the *PARK8* locus. In a small family compatible with ADPD but due to its size only with suggestive linkage to *PARK8*, we identified a heterozygous 205A>G sequence variant leading to an aa exchange from K to E at position 69 in two affected cousins (individuals 21242 and 21243 in Fig. 7A). Two further symptomatic family members deceased and DNA is not available. Two unaffected family members were also tested and do not carry the mutation. Genotyping the 205A>G point mutation in additional 323 sporadic and 38 familial PD patients and in 400 controls did not identify any additional carrier of this mutation. For the two affected cousins mutations were excluded in the *LRRK2* gene (*PARK8* gene) and in 29 other genes of the *PARK8* region (20).

An orthologue of human periphilin was found in mouse, rat, chicken, and *Xenopus*, but not in *Drosophila* and *C. elegans*. The corresponding proteins share 50% (*Xenopus*) to 85% (rat) identity with the human protein. The lysine residue at position 69 is conserved in human, mouse, rat, and chicken, supporting a functional implication of this mutation (Fig. 7B).

Periphilin displays an antiapoptotic function, both under basal conditions and under 6-OHDA treatment

Stable synphilin-1 transfectants or mock-transfected HEK293 cells were transiently transfected with periphilin (wt or mutant) or empty vector and pretreated without or with 6-OHDA. As shown in Fig. 8A (left side), periphilin significantly reduced caspase-3 activity under basal conditions and 6-OHDA treatment ($p<0.05$). For both conditions, no significant differences were found between wt and mutant periphilin transfected cells. Furthermore, synphilin-1 significantly downregulated caspase-3 activity in HEK293 cells both under basal ($p<0.05$) and 6-OHDA-induced ($p<0.001$) conditions, as shown previously (12). In stable synphilin-1 transfectants no synergistic antiapoptotic effect could be observed upon co-transfection of periphilin (Fig. 8A, right side), neither under basal conditions nor under 6-OHDA stimulation and no significant differences were evident between co-transfection of wt and mutant periphilin.

Periphilin is expressed in the murine nervous system

Upon staining of E11.5 heterozygous gene trap embryos using lacZ as reporter for periphilin expression, a ubiquitous staining could be observed (Fig. 9A), in particular in the developing somites and limbs. In the central nervous system, expression was particularly strong in the forebrain and the mid-/hindbrain region. In adult brain, periphilin is also ubiquitously expressed, however, specific regions are most prominently stained such as the cortex, the pyramidal cell layer of the hippocampus, dentate gyrus, striatum, ventral forebrain, subthalamic nucleus, superior colliculus, the granule cell layer of the cerebellum, pons, and the nuclei of the cranial nerves. The dopaminergic neurons of substantia nigra pars compacta and the ventral tegmental area (VTA), identified by tyrosine hydroxylase immunoreactivity, are also strongly stained (Fig. 9B-E).

Compatible with the ubiquitous expression of periphilin during mouse development, extensive genotyping of offspring from animals heterozygous for the periphilin knockout could not detect any homozygous individual among 47 individuals at weaning age, as well as among eight E10.5 and 39 E7.5 embryos. We therefore conclude that the homozygous deficiency of periphilin is lethal in early embryogenesis.

DISCUSSION

Here, we establish periphilin as novel component of the protein network related to PD based on (1) its interaction and co-localization with the α-synuclein interacting protein synphilin-1, (2) its presence in LB, (3) the identification of a missense mutation in two cousins suffering from PD, and (4) the functional implication of periphilin in the control of cell death. We further demonstrate by lacZ reporter gene expression in gene trap mice that (5) periphilin is highly expressed in the substantia nigra, a brain region most severely affected in PD.

The highly insoluble periphilin protein has been described as interactor of periplakin, a protein involved in the differentiation of epidermal keratinozytes (14). Periphilin is found in nuclear granules and at the nuclear membrane of undifferentiated keratinozytes, as well as at the cell periphery and cell-cell junctions of differentiated keratinozytes. PCR amplification from tissue cDNA panels revealed that different isoforms of periphilin are expressed in human brain, kidney, liver, lung, pancreas, placenta, heart, and skeletal muscle (14). We confirmed the expression of periphilin in human brain by cloning periphilin from pooled human brain cDNA, by detecting periphilin in LB of PD patients (Fig. 6A), and by expression analyses of human brain lysates (Fig. 6C). The periphilin isoform studied in this work lacks exons 1, 2, 4, and 12-14 (exon 1 as part of the 5'UTR, the remaining exons due to alternative splicing) and is therefore identical to the keratinozyte-specific isoform described before (14). Other tissue-specific isoforms have been described, arising from alternative splicing of exons 2, 4, 7, and 11-14 (14). The functional differences of these isoforms and how the K69E mutation potentially affects their properties need to be defined in the future.

We confirmed the physical interaction between periphilin and synphilin-1 both *in vitro* and *in vivo*. First, co-immunoprecipitation in combination with SELDI-MS technology verified the interaction of both proteins in a cellular model transiently overexpressing both proteins. This technique has been successfully used to detect protein-protein interactions before (17). Second, we verified that an interaction occurs also between endogenous periphilin and synphilin-1 in a transgenic mouse model overexpressing myc-tagged synphilin-1 (Fig. 4). The interacting domains have been narrowed to aa 557-920 in the synphilin-1 protein and aa 288-313 in the periphilin protein (Fig. 2). The interacting domain of synphilin-1 is therefore different compared to other synphilin-interactors that bind to the central region of synphilin-1, harboring four of the ankyrin-like repeats and a coiled-coil domain. Amino

acids 349-555 have been shown to be necessary and sufficient for the interaction with α-synuclein, and the strongest interaction of parkin has been observed with aa 214-556 of the synphilin-1 protein, for review see (11). Similarly, dorfin interacts with synphilin-1 through its central portion (21). The minimal binding region of the E3 ligases SIAH-1 and SIAH-2, however, is constituted by the first 202 (SIAH-1) or the first 227 (SIAH-2) aa, respectively, as reviewed in (11). The C-terminal part of the synphilin protein, which is necessary and sufficient for the interaction with periphilin, contains the fifth and sixth ankyrin domain and the R621C substitution. This mutation, however, did not cause a differential interaction with periphilin. In the periphilin protein, the C-terminal region is essential for the interaction with synphilin-1. This region contains heptad repeats which potentially form α-helical conformation (14).The final 25 aa of the periphilin protein that are indispensable for the synphilin-periphilin-interaction contain the last of four predicted helices. Above that, the last 110 aa are responsible for the homodimerization of periphilin (14). Deletion of the last 25 aa not only completely abolishes the interaction with synphilin-1 but also this self-interaction. The ability to dimerize might change protein solubility properties which could be a prerequisite for the interaction with synphilin-1.

In support of the periphilin-synphilin-interaction, we found that synphilin-1 and endogenous periphilin co-localize in the cytosol of HEK293 cells (Fig. 5A-C). Periphilin is also present in the cytoplasm of human neuronal cells. Comparably, periphilin was also detected in the cytoplasm of differentiated keratinocytes and in confluent culture of Madin-Darby canine kidney cells overexpressing periphilin (14). It should be stated, that the localization of endogenous periphilin seems to be cell type dependent. For example, in neonatal foreskin, periphilin is localized in the cell periphery of keratinozytes of the granular cell layer, while a prominent nuclear staining is observed throughout the epidermis and in dermal fibroblasts (14). More evidence for a co-localization of periphilin and synphilin-1 *in vivo* comes from the finding that periphilin and synphilin-1 are present in a rat brain synaptosomal fraction (P2) (Fig. 4D). Even though periphilin is not enriched in a crude synaptic vesicle fraction (LP2), we can detect significant levels of periphilin in this fraction, suggesting that periphilin and synphilin-1 may interact at the presynaptic terminal (Fig. 4D). The absence of complete matching in the subcellular distribution of two interacting proteins is not uncommon. For instance, while synphilin-1 is enriched in the LP2 fraction (18), α-synuclein was found to be located mainly in the LS2 fraction of mouse and rat brain (22).

Further supporting the interaction of periphilin and synphilin-1, both proteins are found in LB (Fig. 6A and (23)). Although Fig. 6A shows a positive staining for periphilin mainly in the halo, the core was also slightly stained. In other LB, an even more intense staining of the core could be detected. Conversely, synphillin-1 is found predominantly in the core with less but still visible staining in the halo. Structural or chemical requirements for proteins to become part of the different compartments of a LB are still to be defined. However, as both proteins do occur within the LB, there is at least indirect support of an interaction of the two proteins, even in their aggregated forms.

Mutational studies examining a possible involvement of periphilin in PD revealed a single point mutation causing a K69E substitution in two patients of one small PD family. Two unaffected members of this family do not carry the mutation. The substitution has not been found in a total of 800 chromosomes of control individuals excluding the exchange as a polymorphism. An extended mutation analysis of further 361 PD patients did not reveal an additional mutation carrier suggesting that the K69E mutation is not frequent among PD patients. As the *periphilin* gene maps close to the *PARK8* locus, we also excluded *LRRK2* mutations in the two affected cousins carrying the K69E substitution (data not shown). However, although we have established periphilin as new member of the PD pathway, it still needs to be shown if other PD families carry the K69E mutation or other aberrations in the *periphilin* gene before one can establish periphilin as PD gene. This might be a time intense search as mutations in other PD genes as α-synuclein, PINK1 or DJ-1 are rather rare, e.g. (24-26). Although we did not find any functional evidence that the K69E mutation does influence the interaction with synphilin-1, further studies will have to demonstrate if periphilin interacts with other proteins of the PD pathway and if the mutation alters these interactions.

It is established that synphilin-1 has an antiapoptotic effect in neuronal and HEK293 cells by reducing 6-OHDA- and staurosporine-induced caspase-3 activation (12). This led us to investigate whether periphilin might exert a similar effect on cell death processes and whether it influences the function of the synphilin-1 protein. Indeed, we found that also periphilin significantly reduced caspase-3 activity, both under basal conditions and 6-OHDA treatment (Fig. 8A). Co-transfection of periphilin to stable synphilin-1 transfectants did not result in a synergistic antiapoptotic effect, neither under basal conditions nor under 6-OHDA stimulation (Fig. 8A). The absence of a functional crosstalk between synphilin-1 and periphilin concerning apoptosis could be due to the fact that periphilin might control a

synphilin-dependent function unrelated to the ability to modulate cell death. Since not much is known about other functions of synphilin-1, it can only be speculated that the analysis of another functional paradigm will unmask a functional crosstalk in the future. The K69E mutation does not affect the antiapoptotic phenotype of periphilin. Other cellular functions of periphilin remain to be identified and future analyses will have to show if the K69E mutation alters these functions.

Evidence that periphilin might be involved in a number of processes *in vivo* comes from our studies of mice with an insertion of a gene trapping vector in the *periphilin* gene. Gene trap vectors simultaneously mutate and report the expression of endogenous genes. Mice homozygous for the periphilin knockout die before embryonic day 7.5. This points to an essential and general role during and maybe even before gastrulation. Expression analysis using lacZ as reporter revealed that *periphilin* is widely expressed in the developing mouse embryo with high expression levels in the developing brain, the neuroepithelium of the neural tube, and in somites (Fig. 9). The strong expression in the developing as well as in the adult brain also indicates a function in neuronal physiology. Interestingly, periphilin is highly expressed in dopaminergic neurons of substantia nigra pars compacta and the ventral tegmental area, brain regions severely affected in PD. The definition of novel functions and properties of periphilin both under physiological and pathological conditions beyond the ones described in this paper are subject to future research.

MATERIALS AND METHODS

Yeast two-hybrid assays and yeast culture

The bait construct for Y2H screening was generated as fusion construct encoding aa 177-348 and 557-920 of synphilin-1 in the pLEXA-DIR vector (Dualsystems Biotech AG, Switzerland). The central ankyrin-like repeats, the coiled-coil domain, and the ATP/GTP-binding domain had been omitted to avoid unspecific interactions. The first 176 aa of synphilin-1 were truncated since they caused self-activation of the 5' construct due to their acidic nature (27). Self-activation was assayed by co-transformation of the bait together with a control prey. The bait construct was co-transformed with a human brain cDNA library into yeast strain L40 (MATa his3_200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4) using standard procedures (28). Positive transformants were tested for β-galactosidase activity using a filter assay (29). Library plasmids were isolated from positive clones and retransformed into L40 with (a) the bait plasmid and (b) a control bait encoding a LexA-lamin C fusion. Positives that showed β-galactosidase activity when co-expressed with the bait but not when co-expressed with the control bait were considered to be bait dependent positive interactors. The identity of positive interactors was determined by sequencing.

For pairwise interaction tests, plasmids encoding periphilin-GAL4 and 5' (aa 177-348) or 3' (aa 557-920) portions of synphilin-1 fused to a LexA binding domain were generated using *SalI* and *NotI* restriction sites of pBTM117c (30) and pGAD426 (kindly provided by Erich Wanker, MDC Berlin, Germany). The prey vector pGAD426 containing an upstream GAL4 activation domain had been modified from pGAD424 (BD Biosciences Clontech, Germany). The cloning of periphilin from pooled human brain cDNA is described in the following subsection. The periphilin K69E mutation was inserted using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, The Netherlands). Primer sequences for cloning and sequencing are available from the authors. The generation of periphilin deletion constructs has been described (14). Constructs referred to as encoding aa 87-374, 197-374, and 197-349 in this former publication correspond to encoded aa 25-313, 136-313, and 136-287 in our numbering, respectively. Plasmids were co-transformed into L40 and grown at 30°C for 3 days on synthetic media without leucine and tryptophan. The β-galactosidase reporter activity was assayed on nitrocellulose filters by incubating freeze-fractured colonies in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 34.6 mM β-mercaptoethanol and 0.65 mM X-gal at 37°C for 15 min to 19 hr. Self-activation was assayed as described above.

Cloning of periphilin cDNA

Periphilin was amplified from cDNA obtained from human brain by RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). To allow the amplification of all known splice variants, a 5' primer complementary to the 5'UTR upstream of the first splice site was chosen. Similarly, an unspecific T7-$(dT)_{24}$ primer was used to facilitate the amplification of all periphilin splice variants. The polymerase chain reaction (PCR) was carried out using the Expand Long Template PCR System (Roche, Germany) according to the manufacturer's instructions. The amplicon was purified with the QIAquick Gel Extraction Kit (QIAGEN, Germany) and 5' and 3' terminal sequences were sequenced. Subsequently, primer pairs containing *SalI* and *NotI* restriction sites were designed to facilitate cloning into Y2H bait- and prey plasmids. Primer sequences are available from the authors on request.

V5-tagged expression constructs

To prepare C-terminally V5-tagged periphilin expression constructs, periphilin was amplified via PCR from full-length periphilin in pBTM117c. The applied primer pairs incorporated *HindIII* and *XhoI* restriction sites to facilitate subcloning in the expression plasmid pcDNA3.1/V5-His®A (Invitrogen, Germany), primer sequences are available on request. The insert DNA was sequenced to obviate PCR-generated errors.

Cell culture and transfection

HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Germany) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptavidin in a 5% CO_2 atmosphere. For transient transfections, cells were plated on 6-well plates and transfected with Lipofectamine (Invitrogen, Germany) in a ratio of 2 µg DNA to 5 µl Lipofectamine.

Surface enhanced laser desorption ionization-mass spectrometry (SELDI-MS)

HEK293 cells were transiently transfected with 2 µg of V5-tagged wt periphilin and/or FLAG-tagged wt synphilin using Lipofectamine (Invitrogen, Germany). FLAG-tagged wt synphilin-1 constructs were subcloned from the pAd-Track-CMV-FLAG-R621 synphilin-1 vector (described by Marx and coworkers (2003)) into pcDNA3.1 vector using *Xho*I and *Hind*III sites.

The protein-protein interaction assay and coupling of unspecific antibodies to IDM beads (Ciphergen Biosystems Ltd., Fremont, CA) as negative control was performed as described (17). Agarose conjugated V5 antibodies were purchased from Sigma, Germany, and used to specifically immunoprecipitate V5-tagged periphilin from 130 µl of cell extract. Bound proteins were eluted with 10 µl 50% acetonitrile/0.5% trifluoroacetic acid (TFA). Five µl of the eluted samples were applied to the activated reverse phase surface of an NP20 ProteinChip array (Ciphergen Biosystem Inc., Fremont, CA) and dried on air. After washing with 3 µl aqua bidest, 0.5 µl of sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) were applied twice and the array was analyzed in a ProteinChip reader series 4000 mass spectrometer (Ciphergen, Fremont, CA).

For the analysis of fragment masses, proteins eluted from IDM beads or agarose conjugated V5 tag antibodies were tryptic digested as previously described (17). The digest products were spotted on NP20 ProteinChip arrays and peptide fragment masses were determined by the ProteinChip reader series 4000 instrument. Theoretical tryptic digests were performed using the Expasy PeptideMass tool (http://us.expasy.org/tools/peptide-mass.html). Theoretical fragments were marked in the spectra, assuring that these peaks were not present in approaches using non-specific antibodies, and were used to identify the associated proteins with a public database (http://129.85.19.192/profound_bin/WebProFound.exe).

In an immuno-deplete assay, agarose conjugated V5 tag antibodies were washed twice with a buffer containing 20 mM Hepes (pH 7.8), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% NP-40 and afterwards incubated with 5 µl of a lysate from HEK293 cells transiently transfected with 2 µg of V5-tagged wt periphilin and 2 µg of FLAG-tagged synphilin-1. As a negative control, 5 µl of the lysate were incubated with IDM beads without the specific antibody for 45 min on ice. Then, samples were cleared by centrifugation and 3 µl of each supernatant were analyzed by ProteinChip Arrays.

Peptide conjugates and antibody generation

The antigenic peptide RSKAIASKTKEIEQVYRQD was selected from the protein sequence using the lasergene software (DNASTAR, Madison, WI). It was synthesized as single peptide and as multiple antigen peptide (MAP) $(RSKAIASKTKEIEQVYRQD)_8$-$(Lys)_4$-$(Lys)_2$-Lys-Gly-OH (31) using standard Fmoc/tBu chemistry (32) on a multiple peptide synthesizer Syro II (MultiSynTech, Germany). The peptides were purified using reversed phase high performance liquid chromatography (RP-HPLC) and the identity was confirmed using electrospray ionization mass spectrometry (ESI-MS). Peptide purities

were determined via analytical HPLC and proved to be > 90%. The single peptide was coupled to keyhole limpet hemocyanin (KLH) using the glutardialdehyde method.

The antiserum (1313/1) was obtained after repeated immunization of a rabbit with a 1:1 mixture of the peptide-KLH-conjugate and the MAP and further purified by affinity chromatography on a CH activated Sepharose 4B (GE healthcare, Germany) containing the immobilized peptide via a stable peptide bond. The antiserum was applied onto the column at 0.5ml/min and recycled overnight. The column was washed with 20 column volumes of PBS. Elution was performed with 10 volumes of 0.1 M glycine/HCl (pH 2.5). Antibody containing fractions were immediately neutralized with 1 M Tris/HCl (pH 8.5) and concentrated on a 20 kD membrane. The resulting antibody was retested in an enzyme linked immunosorbent assay (ELISA) and showed the expected specificity.

Solubilization of periphilin protein

To extract V5-tagged periphilin from cell cultures, transiently transfected HEK293 cells were washed 48 hr after transfection with cold PBS and harvested with a radioimmunoprecipitation (RIPA-) buffer as described elsewhere (14).

Periphilin extraction from postmortem tissue: The brains from one male who died of PD at the age of 63 and one male who died from cardiorespiratory insufficiency at the age 59 were obtained from a brain bank established at the Institute of Brain Research at the University of Tuebingen, Germany. Both individuals signed an informed consent. To proof the interaction of endogenous periphilin with synphilin-1, the cortex of an adult mouse transgenic for myc-tagged synphilin-1 was dissected. A small piece of human frontal cortex and the entire murine cortex were thawed on ice and 1 ml (for the human sample) or 500 µl (for the murine sample) TES buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8.0, 100 mM NaCl; protease inhibitor Complete, Roche, Germany) were added. The tissue was chopped up with an UltraTurrax® (IKA Works, Inc., Wilmington, NC). Subsequently, 100 µl of TES containing 10% NP-40 (IGEPAL CA-630, Sigma, Germany) were added. The samples were mixed and incubated on ice for 40 min. After centrifugation (16.100 *g*, 10 min, 4°C), the supernatant was harvested and centrifuged again for 20 min under the same conditions. The supernatant was frozen at –80°C under addition of 10% glycerol (Roth, Germany).

Immunoprecipitation

Transiently transfected cells were extracted 48 hr after transfection or postmortem tissue was lysed as described above. Precipitation was carried out according to manufacturer's instructions using anti-V5 Agarose affinity gel (Sigma, Germany) for the precipitation of V5-tagged periphilin from lysates of HEK293 cells or anti-c-Myc Agarose affinity gel (Sigma, Germany) for the precipitation of myc-tagged synphilin-1 from the cortex lysate of a synphilin-1 transgenic mouse.

Western blot analysis

For Western Blotting, 50 µg of cell or tissue lysates or supernatants from immunoprecipitation experiments (see above) were separated through SDS-PAGE and transferred to nylon membranes. The membranes were blocked for 2 hr at room temperature (RT) in 5% nonfat dry milk powder in Tris-Buffered Saline (TBS). After incubation with rabbit anti-periphilin antibodies (1313/1; 1:1000 in TBS with 5% nonfat dry milk powder) or mouse anti-myc antibodies (1:200, Santa Cruz, Germany) for 2 hr at RT, the membranes were washed three times with TBS containing 0.1% Tween-20 (TBST), incubated for 1 hr at RT with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, 1:3000; anti-mouse, 1:2500; Amersham Biosciences, Germany), washed again three times with TBST and once with TBS, and developed using ECL™ Western blotting Detection Reagents (Amersham Biosciences, Germany).

Immunofluorecence microscopy

HEK293 cells were grown on Poly-L-Lysine coated slides and transfected with 2 µg of FLAG-tagged wt synphilin-1 using Lipofectamine (Invitrogen, Germany) in a ratio of 2 µg DNA to 5 µl Lipofectamine. Thirty hours after transfection, cells were washed with PBS, fixed with 4% paraformaldehyde (PFA), and permeabilized with 100% methanol. After washing with PBS, cells were incubated for 30 min in 10% normal donkey serum (NDS) in PBS. Then, cells were labeled overnight at 4°C with rabbit 1313/1 periphilin antibody (1:80) and mouse anti-FLAG monoclonal antibody (Sigma, Germany, 1:500) in PBS containing 5% NDS. Next day, cells were washed with PBS and labeled with Cy2-conjugated anti-rabbit (1:300) and Cy3-conjugated anti-mouse (1:500) secondary antibodies (Dianova, Germany). The slides were analyzed under a laser scanning microscope (LSM 510 Meta, Zeiss, Germany). The images were stored and processed with the LSM Image Browser (Zeiss, Germany).

Subcellular fractionation

Subcellular fractionations of homogenates from 5 adult rat brains were carried out as described (18) (H, homogenate; S1, supernatant of the homogenate at low-speed centrifugations and corresponding pellet P1; P2, crude synaptosomes pellet of S1 and corresponding supernatant S2 at medium-speed centrifugations; P3, pellet of S2 and corresponding supernatant S3 at high-speed centrifugations; LP1, pellet obtained after hypotonic lysis of P2 and corresponding supernatant LS1; and LP2, vesicle-enriched pellet of LS1 and corresponding supernatant LS2). Protein samples (50 µg) were denatured for 5 min at 100°C in SDS sample buffer and analyzed by Western blot using an anti-periphilin antibody (1313/1) and anti-synphilin-1 antibody (10). Periphilin and synphilin-1 steady-state levels were determined by densitometry using ImageJ program.

Immunohistochemistry of adult human brain

Staining for LB was carried out on formalin-fixed, paraffin-embedded 4 µm-thick sections using the avidin-biotin-immunoperoxidase technique. Sections were deparaffinized and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 0.05% TBS for 20 min. After washing in 0.05% TBS, sections were brought to 5% bovine serum albumine (GibcoBrl, Grand Island N.Y., USA) and 0.3% Triton X-100 (Biochemica, Germany) for 1 hr at RT. Sections were then incubated with the affinity-purified rabbit anti-human periphilin antibody (1313/1, see section antibody generation), diluted 1:10 in 0.05% TBS containing 3% bovine serum albumin overnight at 4°C. Next day, sections were washed with 0.05% TBS, incubated in biotinylated goat anti-rabbit IgG (StrAviGen multi-Link Kit, BioGenex, San Ramon, CA) diluted 1:200 in 0.05% TBS for 1 hr at 4°C, rinsed in 0.05% TBS, and then incubated for 45 min at 4°C in StreptABComplex/HRP (Dakocytomation, Glostrup, Denmark). The enzymatic reaction was carried out with 4% 3-amino-9-ethylcarbazole (ACE, BioGenex, San Ramon, CA). Sections were counterstained with hematoxylin for 20 sec. To confirm specificity of the immunostaining, the periphilin antibody was preabsorbed overnight with peptide solution (2 mg peptide/ml in 0.05% TBS) and used to stain a control section.

Mutation analysis

Exon sequences and exon-intron-boundaries from eight index patients of four families with PD were amplified and sequenced with the BigDyeTerminator Cycle sequencing kit 3.1 (ABI, Germany) on an ABI 3730 sequencer. Primer sequences are available from the authors on request. Two of these

families had been linked to the PARK8 locus and had been previously characterized clinically and pathologically (33). Two other small families showed weak linkage to *PARK8* due to their size, one of them being the family where we identified the periphilin mutation. In order to confirm the detected 205A>G point mutation, additional 323 sporadic and 38 familial PD patients from clinical centers in Munich and Tuebingen were screened. In all patients, PD was diagnosed clinically by specialists in movement disorders according to the UK Parkinson´s Disease Society Brain Bank criteria. After obtaining informed consent, blood samples were drawn for DNA extraction. These PD patients had a male:female ratio of 1.38 and showed a median age at onset of 55,4 ± 19,1 years. 400 healthy, age and sex matched individuals from a population-based cohort panel (Cooperative Health Research in the Region of Augsburg, Germany, KORA S2000 project) served as controls.

Caspase-3 activity measurements

Stable synphilin-1 transfectants or mock-transfected HEK293 cells were transiently transfected with wt or mutant periphilin or empty pcDNA3.1/V5-His®A vector as negative control and preincubated without or with 200 µM 6-OHDA for 6 hr, and then caspase-3-like activity was fluorimetrically measured as detailed in (12). Caspase-3-like activity is considered as the Ac-DEVD-CHO-sensitive Ac-DEVD-7-amino-4-methylcoumarin-hydrolyzing activity.

Generation of periphilin deficient mice (KO mice)

A mouse line carrying a mutation in the periphilin gene was generated at the National Research Center for Environment and Health (GSF) in Neuherberg, Germany, a member of the German Genetrap Consortium (GGTC) by taking advantage of the gene trapping method (34). The embryonic stem cell (ES cell) clone A043F08 was generated using the vector pT1betageo (35). Integration of this vector into an intron, exon or the 5´ UTR of an expressed gene results in a lacZ/neomycin (®geo) fusion transcript leading to a dysfunction of the tagged gene (35). By 5' rapid amplification of cDNA ends (RACE) and sequencing, the gene trap clone A043F08 was found to carry a vector insertion in intron 6 of the mouse periphilin-1 gene. ES cells carrying this periphilin mutation were injected into C57BL/6 host blastocysts to generate chimeras. The agouti coat color marker was used to assess germline transmission, which was verified with internal and external probes in Southern blot analyses. After germline transmission, the mouse line designated Pphln1 GT(pT1Betageo)6Flo was bred to homozygosity to evaluate whether the absence of periphilin-1 still allows viability of the mutants. A

PCR genotyping assay allowed to distinguish homozygous and heterozygous embryos. Primer sequences are available from the authors on request.

Immunohistochemistry and lacZ staining of the developing mouse embryo and the adult mouse brain

Tyrosine hydroxylase (TH) immunohistochemistry: 6 months old wt mice (n=2) were perfused using 4% PFA in 0.1 M PBS, post-fixed in the same solution for 2 hr, and cryoprotected in 25% sucrose (w/v) in 0.1 PBS overnight. The entire brain was cut on a microtome into 40 µm sections and stored in 0.1 M PBS. After inactivation of endogenous peroxidase (10 min in 0.1% H_2O_2 in 0.1 M PBS), washing in 0.1 M PBS, and preincubation in 2% FCS in 0.1 M PBS/0.2% Triton, sections were incubated overnight in rabbit anti-TH antiserum in 0.1 M PBS (4°C; 1:1000; PelFreeze, Rogers, AR). Thereafter, the sections were washed 5 x 10 min in 0.1 M PBS/0.2% Triton and incubated for 1 hr in biotin-SP conjugated goat anti-rabbit antiserum in 0.1 M PBS (RT; 1:300; Jackson ImmunoResearch, United Kingdom). Then, the sections were washed again 5 x 10 min before being incubated in peroxidase-conjugated biotin complex solution in 0.1 M PBS (45 min; RT; Vector Laboratories Inc., Burlingame CA). Sections were rinsed 2 x 10 min in 0.1 M PBS, 2 x 10 min in 0.1 M Tris-HCl and then stained using 0.05 M DAB/0.025% H_2O_2 in 0.1 M Tris-HCl. After washing in 0.1 M PBS, the sections were mounted, dehydrated, and coverslipped in Rotihisto-Kit (Roth, Germany).

LacZ histochemistry was performed on whole E11.5 embryos and on brain sections of 3 month-old heterozygous Pphln1 GT(pT1Betageo)6Flo mice (n=6). Whole brains were dissected from PFA-perfused mice, fixed, and cut on a vibratom into 300 µm thick sections. Whole embryos and brain sections were stained in 0.1% X-gal, 0.005 M potassium-ferrocyanide and 0.005 M potassium-ferricyanide at 30^{o}C for 12-16 hr (all components were purchased from Sigma, Germany). Photographs were taken using an Axioplan microscope from Zeiss, Germany.

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CONFLICT OF INTEREST STATEMENT

None declared.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Schematic presentation of periphilin and synphilin-1 cDNA. (A) The box represents full-length periphilin cDNA with its 14 exons. The exons lacking in the coding sequence (CDS) under investigation due to alternative splicing are represented by grey boxes. Exon 1 is part of the 5'UTR and is represented by a striped pattern. The arrowhead indicates the newly identified mutation K69E in exon 6 of the periphilin cDNA. Lines represent the fragments of periphilin used as prey in pairwise Y2H interaction tests. Amino acids are numbered consecutively from the first translated in frame start codon in exon 3, skipping the amino acids of exon 4 deleted by alternative splicing. (B) The synphilin-1 cDNA is represented as box. The ankyrin-like repeats, the coiled-coil-domain, the ATP/GTP-binding domain and the R621C substitution are indicated. Lines mark the synphilin-1 fragments used as bait in pairwise Y2H interaction tests.

Figure 2. Molecular interaction of periphilin and synphilin-1. (A) Wild type and K69E mutant periphilin, used as Y2H-prey, interact with aa 557-920 of synphilin-1 (fused to a LexA binding domain). The interaction is not abolished by the R621C mutation of synphilin-1. A set of N-terminal deletions of periphilin uncovered that aa 288-313 of the periphilin protein are indispensable for the periphilin-synphilin-1-interaction, because this truncation is the only modification abrogating the interaction. The mapping approach was repeated with synphilin-1 R621C aa 557-920 and yielded the same results (not shown). Each bait- and prey construct was tested for self-activation by co-transformation of an empty prey or bait vector, respectively, as shown in columns 2 and 3. (B) Schematical representation of the molecular interaction of periphilin and synphilin-1.

Figure 3. Confirmation of the interaction between periphilin and synphilin-1 applying SELDI-MS ProteinChip technology. (A) V5 antibodies conjugated to agarose beads were used to specifically immunoprecipitate V5-tagged periphilin from lysates of HEK cells transiently transfected with V5-periphilin and FLAG-synphilin-1. Bound proteins were eluted and analyzed by SELDI-MS. Using anti-V5 coupled beads (upper panel) compared to an approach using a non-specific antibody (bottom panel) we detected specific signals at approximately 101.2 kD as well as 39.5 kD corresponding very well to the calculated molecular mass of FLAG-tagged synphilin (101.5 kD) and V5-tagged periphilin (39.5 kD). (B) For protein identification, eluted proteins that co-immunoprecipitated with V5-tagged

periphilin were subjected to tryptic digestion. In this way generated specific peptide mass fingerprints (PMF) that corresponded to theoretical periphilin or synphilin-1 tryptic digest products were marked as specified in Table 1 (upper panel for synphilin-1, middle panel for periphilin). As negative control, protein eluate from the protein-protein interaction assay using an unspecific antibody was tryptic digested and no specific PMF corresponding to periphilin or synphilin-1 occurred (bottom panel). (C, D) Confirmation of the periphilin-synphilin interaction applying an immuno-depletion assay. Hereby, a lysate derived from HEK cells transiently transfected with V5-tagged periphilin and FLAG-tagged synphilin-1 was immunoprecipitated with anti-V5 conjugated agarose beads. Analysis by ProteinChip reader mass spectrometry revealed that the signals corresponding to periphilin (C, upper panel) as well as to synphilin-1 (D, upper panel) were depleted in the supernatant. In a negative control using a non-specific antibody both signals were clearly detectable (bottom panels in C and D).

Figure 4. Interaction of endogenous periphilin and synphilin-1 in a mouse model transgenic for synphilin-1. Synphilin-1 was immunoprecipitated from cortex lysate of a mouse transgenic for human myc-tagged synphilin-1 using agarose-conjugated c-Myc antibodies. Subsequently, synphilin-1 and periphilin immunoreactivity was monitored applying myc- or anti-periphilin (1313/1) antibodies, respectively. The figure shows that periphilin specifically co-immunopreciptated with synphilin-1. As negative control, the same cortex lysate was immunoprecipitated with agarose-conjugated V5 antibodies, which yielded neither a signal for synphilin-1 nor periphilin.

Figure 5. Periphilin and synphilin-1 display co-localization in cell culture and adult rat brain. (A) Endogenous periphilin is expressed in the cytosol of HEK293 cells, as determined by staining with an antibody specific for periphilin (1313/1). (B) HEK293 cells transiently transfected with wt FLAG synphilin-1 were labeled with anti-FLAG antibodies (green). (C) The confocal sections demonstrate that both synphilin-1 and endogenous periphilin are co-localizing in the cytosol. Scale bar, 10 μm. (D) Periphilin and synphilin-1 are present in a synaptosomal fraction (P2) and in crude synaptic vesicle fractions (LP1 and LP2). Subcellular fractions were obtained and designated as described in Materials and Methods. Expression levels of periphilin in rat brain fractions (50 μg) were determined by Western blot using an anti-periphilin antibody (bottom panel). The upper panel shows the distribution of synphilin-1 (50 μg) in the same fractions as periphilin as determined by Western blot using an anti-synphilin-1 antibody (Engelender et al., 1999). (E) Quantitative densitometric analysis of periphilin and

synphilin-1 immunoreactivities recovered in *D*. The values are normalized to the periphilin or synphilin-1 expression in the homogenate, respectively. The numbers above the bars represent the ratios: normalized periphilin expression/normalized synphilin-1 expression.

Figure 6. Periphilin expression in human brain. (A) Staining of two Lewy bodies (LB) in a neuromelanin (NM) containing neuron using an antibody directed against periphilin. The immunoreacticity is most prominent in the halo, but also visible in the core. (B) Neither staining of the LB nor any other structures is visible after preabsorbtion of the primary antibody. (C) Periphilin is expressed in the postmortem cortex of a PD patient and a control individual with no obvious difference regarding the expression intensity and no indication for differences in posttranslational processing.

Figure 7. Periphilin mutation in two affecteds with autosomal dominant PD and conservation of the wild type K69 residue across species. (A) Electropherograms of the two affected cousins (individuals 21242, 21243) of family M3 with the heterozygous 205A>G mutation leading to the amino acid exchange K69E. (B) Amino acid sequence alignment of periphilin. The alignment was generated with ClustalW. Human (Hs), mouse (Mm), rat (Rn), *Gallus gallus* (Gg), and *Xenopus laevis* (Xl) share between 85% (human/rat) and 50% (human/*Xenopus*) identities. No homologues were detected in *Drosophila* and *C. elegans*. Identities through all species are shown in red, the position of the mutation is indicated in green. The wild type residue lysine (K) is conserved in mouse, rat, and *Gallus*. In *Xenopus* it is replaced by the similar amino acid arginine.

Figure 8. Periphilin reduces basal and 6-OHDA-induced levels of caspase-3 activity. (A) Stably expressing mock-transfected or synphilin-1-transfected HEK293 cells were transiently co-transfected with wt or mutant periphilin or empty vector and treated without or with 200 µM 6-OHDA for 6 hr. Subsequently, caspase-3 activity was monitored. (B) Immunological profile of synphilin-1, periphilin, and tubulin in stable mock-transfected and wt synphilin-1 expressing HEK293 cells transiently co-transfected with wt or mutant periphilin or empty vector. Note that synphilin-1 is cleaved by caspase-3 activity which is increased in response to 6-OHDA stimulation. Therefore, synphilin-1 expression is decreased under 6-OHDA treatment. *Bars*, means ± SD of three independent experiments performed in triplicate (N=9). ***, $p<0.001$; **, $p<0.005$; *, $p<0.05$; ns, nonsignificant. Asterisks above bars

indicate siginficance levels with respect to HEK Mock plus pcDNA under basal conditions. One arbitrary unit (U) corresponds to the release of 4 nmol of 7-amino-4-methylcoumarin.

Figure 9. Periphilin expression in the developing mouse embryo and the adult mouse brain as monitored by beta-galactosidase activity in heterozygous genetrap mutants. (A) High beta-galactosidase activity (blue) is detected ubiquitously in a heterozygous E11.5 genetrap mutant embryo. (B) Detection of beta-galactosidase activity (blue) in a midsagittal brain section of a 3-month-old heterozygous genetrap mutant. Strong activity is observed in the olfactory bulb, cortex, striatum, substantia nigra, and the VTA. (C) Detection of TH-immunoreactivity (brown) and beta-galactosidase activity (blue) in a horizontal section of a 3-month-old heterozygous genetrap mutant. (D,E) High power magnification of the substantia nigra and VTA stained for beta-galactosidase activity and TH-immunoreactivity, respectively. Note the strong beta-galactosidase activity in the region of positive TH-staining.

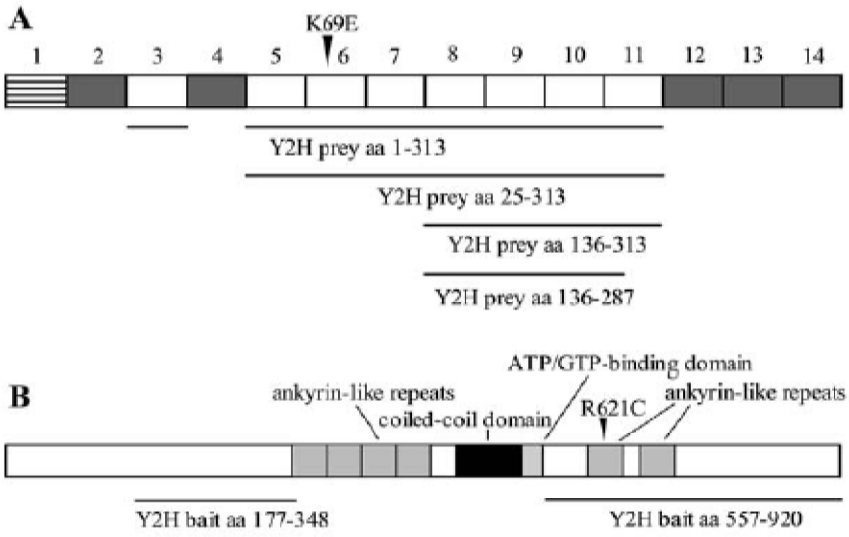
Abbreviations: fb = forebrain; mb = midbrain; hb = hindbrain; sc = spinal cord; drg = dorsal root ganglia; cx = cerebral cortex; str = striatum; th = thalamus; hip = hippocampus; STh = subthalamic nucleus; VTA = ventral tegmental area; SN = substantia nigra; pn = pons; SuC = superior colliculus; IC = inferior colliculus; Teg = tegmentum; Sol = solitary tract; cb = cerebellum; ob = olfactory bulb

TABLES

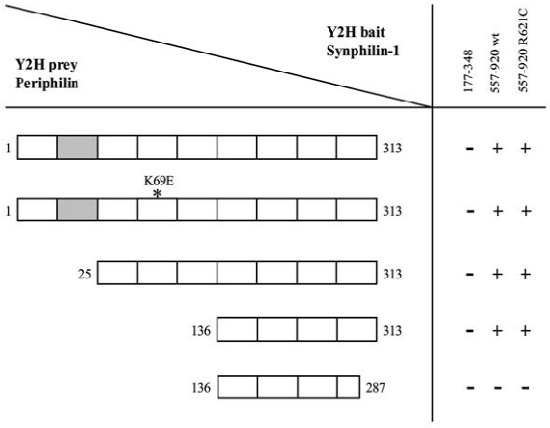
Table 1. Matched peptide mass fingerprints (PMF) used for identification of periphilin and synphilin-1 tryptic digest products.

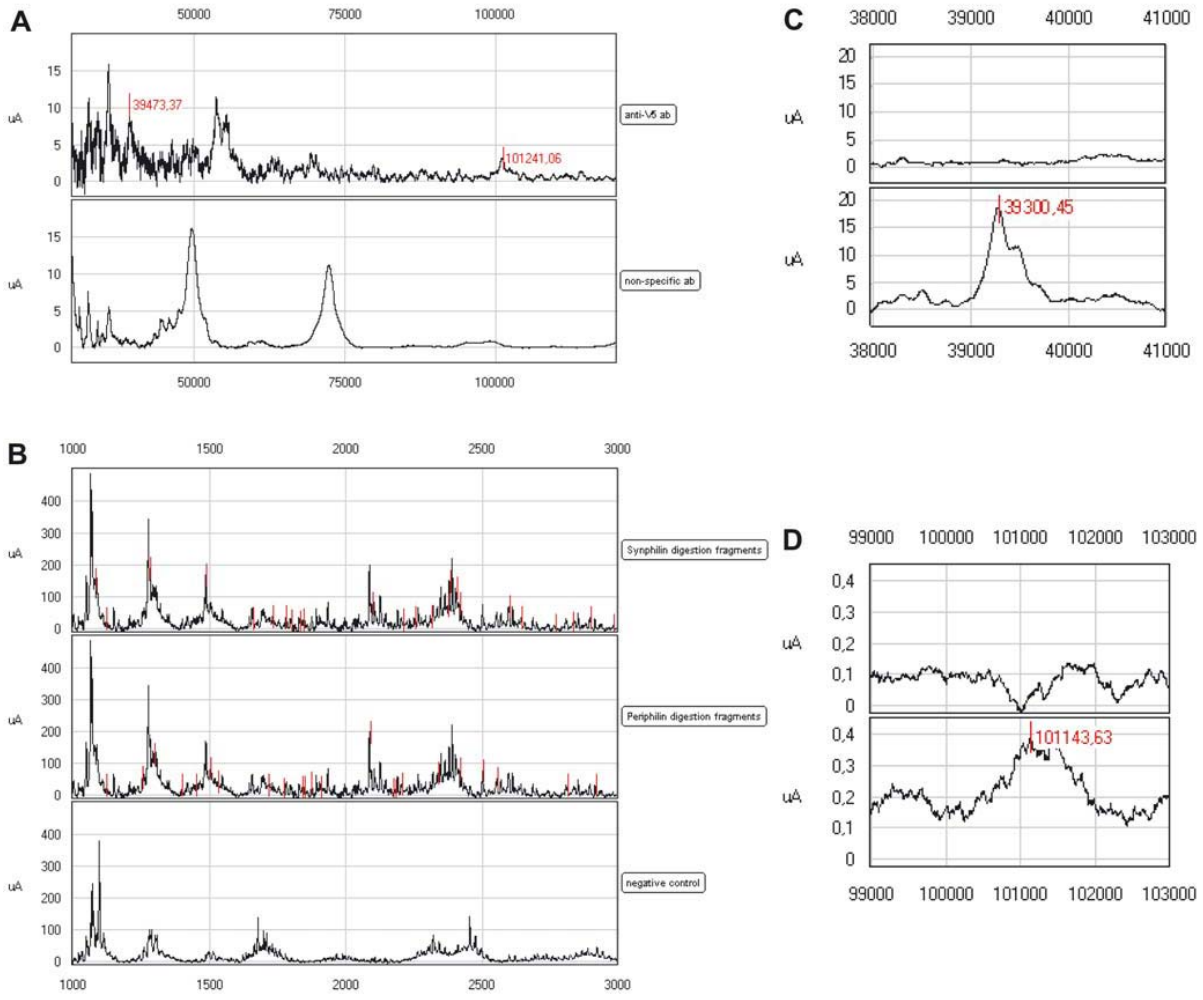
Periphilin			Synphilin-1		
Measured peptide mass	Theoretical peptide mass	Peptide sequence	Measured peptide mass	Theoretical peptide mass	Peptide sequence
771.042	771.872	ESPVGRK	1082.004	1082.231	KHTLASGGRR
786.125	786.756	DDHSASR	1124.795	1125.377	IPLEKRELK
825.149	825.880	KDSPHSR	1283.689	1284.595	LARLRQLMQR
933.807	933.116	SKAIASKTK	1488.907	1488.667	SPSSKRRTSQNLK
1124.795	1125.249	YEYERIPR	1661.513	1661.838	DFLNKTFSDPHGRK
1300.588	1300.481	ERPVQSLKTSR	1661.513	1660.805	TDAKGNPASSASKGKNK
1402.318	1401.547	RKSFYSSHYAR	1783.697	1783.783	HQPETLENNESDDQK
1402.318	1402.489	SFYSSHYARER	1783.697	1783.125	LTPAGLAIKNGQLECVR
1451.679	1451.562	ESPVGRKDSPHSR	1783.697	1784.041	WMVSETEAIAELSCSK
1531.371	1530.663	KSFYSSHYARER	1802.288	1802.113	KHTLASGGRRFPFSIK
1715.928	1715.889	ERSPYKRDNTFFR	1833.705	1834.059	EGQISLLPHLAADNLDK
1774.953	1774.004	ELAEAASKWAAEKLEK	1845.108	1844.953	SEGKSLPSSPSSPSSPASR
1840.685	1840.930	ERAPPRSHPSDESGYR	2098.591	2098.260	TSTSNESGDQLKRPFGAFR
1876.450	1877.052	DGFRRKSFYSSHYAR	2211.265	2211.716	YLVVVETCMSLASQVVKLTK
2091.728	2092.248	DTSPSSGSAVSSSKVLDKPSR	2254.936	2255.624	NTEKLTPAGLAIKNGQLECVR
2174.086	2173.377	KSVRPGASYKRQNEGNPER	2315.143	2315.595	TFSDPHGRKVEKTTPDCQLR
2184.283	2184.443	SPYKRDNTFFRESPVGRK	2376.878	2376.672	ACSTGSSESSSSNMAPFCVLSPVK
2207.517	2207.394	IPRERAPPRSHPSDESGYR	2406.957	2407.487	SLSESDTDSNNSEDPKTTPVRK
2340.994	2341.572	ERSPYKRDNTFFRESPVGR	2419.905	2419.692	VTFEEPVVQMEQPSLELNGEK
2506.201	2506.770	SYSFHQSQHRKSVRPGASYKR	2602.613	2602.026	SILNIVKEGQISLLPHLAADNLDK
2558.146	2557.789	RDEMWSEGRYEYERIPRER	2647.479	2646.955	VTFEEPVVQMEQPSLELNGEKDK
			2768.449	2768.275	QGHTLCSRYLVVVETCMSLASQVVK
			2835.539	2835.040	RVSPLKHQPETLENNESDDQKNQK

Table 1. Lysates of HEK cells transiently transfected with V5-periphilin and FLAG-synphilin-1 were immunoprecipitated using agarose-conjugated V5 antibodies. The eluted proteins were subjected to tryptic digestion and the size of the so obtained peptide mass fingerprints (PMF) was determined by SELDI analysis (first and forth column). In parallel, a theoretical tryptic digestion of synphilin-1 and periphilin was carried out using a public database (Expasy PeptideMass tool; http://us.expasy.org/tools/peptide-mass.html). The PMFs that corresponded to the theoretical synphilin-1 and periphilin tryptic digest products were marked as shown in Figure 3B. The matched theoretical peptide masses are listed in the second and fifth column together with the corresponding peptide sequences in columns 3 and 6. A database query (Profound; http://129.85.19.192/profound_bin/WebProFound.exe) with the matched synphilin-1 PMFs revealed synphilin-1 as interactor of periphilin. The fragments covered 28% of the synphilin-1 protein. Periphilin was also identified in the immunoprecipitates with a coverage of 41%.



Interaction between periphilin (wt or K69E) and synphilin-1 wt aa 557-920			
	Periphilin wt + synphilin 3' wt	Periphilin wt + pBTM117c	Synphilin 3' wt + pGAD426
	Periphilin K69E + synphilin 3' wt	Periphilin K69E + pBTM117c	Synphilin 3' wt + pGAD426
Interaction between periphilin (wt or K69E) and synphilin-1 R621C aa 557-920			
	Periphilin wt + synphilin 3' R621C	Periphilin wt + pBTM117c	Synphilin 3' R621C + pGAD426
	Periphilin K69E + synphilin 3' R621C	Periphilin K69E + pBTM117c	Synphilin 3' R621C + pGAD426
Mapping of the interacting periphilin domain (test with synphilin-1 wt aa 557-920)			
	Synphilin 3' wt + periphilin wt 25-313	Synphilin 3' wt + pGAD424	Periphilin wt 25-313 + pBTM117c
	Synphilin 3' wt + periphilin wt 136-313	Synphilin 3' wt + pGAD424	Periphilin wt 136-313 + pBTM117c
	Synphilin 3' wt + periphilin wt 136-287	Synphilin 3' wt + pGAD424	Periphilin wt 136-287 + pBTM117c

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Periphilin digestion fragments
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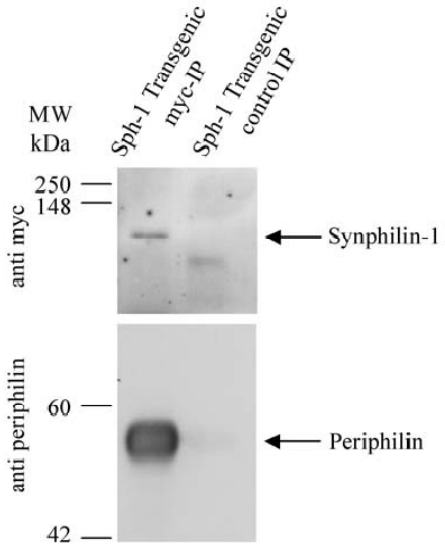
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Synphilin-1
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Periphilin
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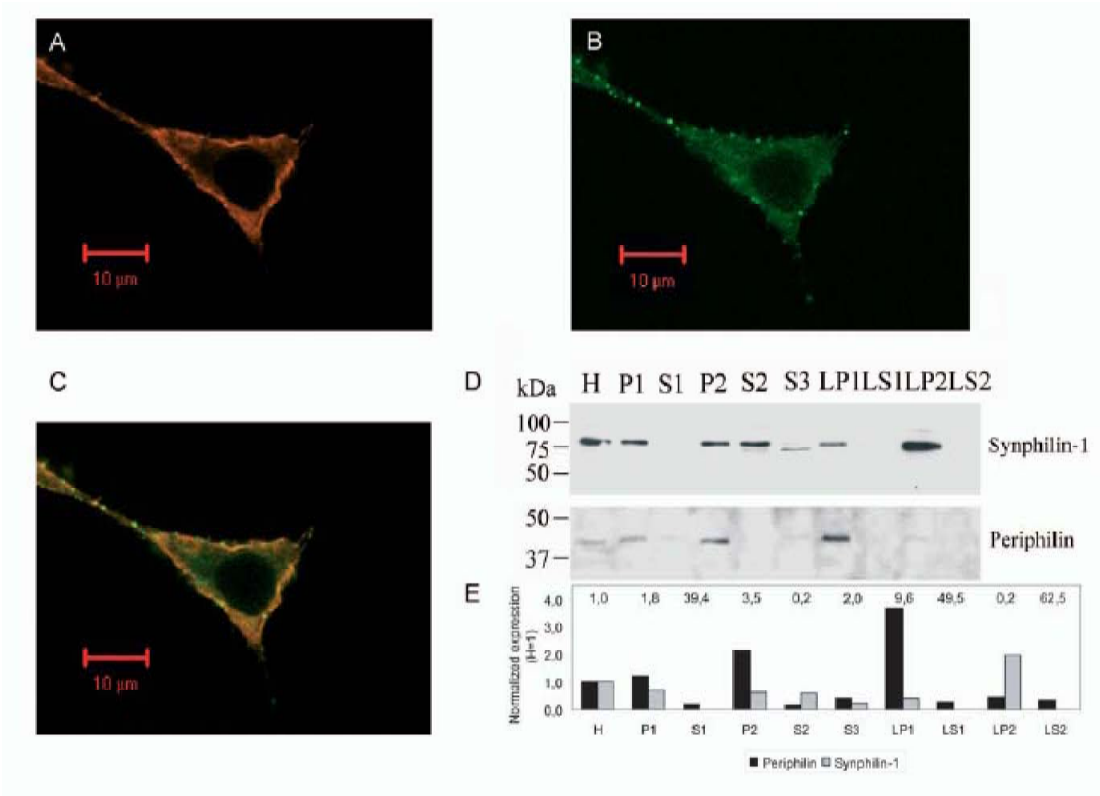
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Synphilin-1
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Periphilin
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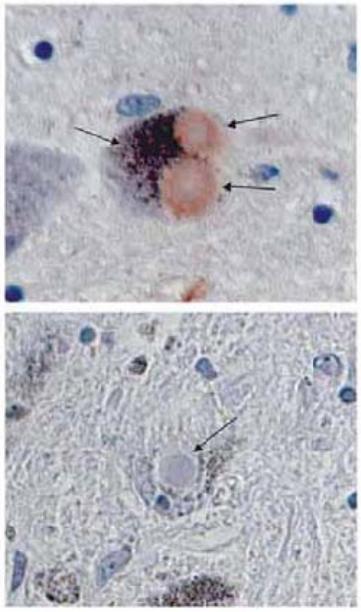
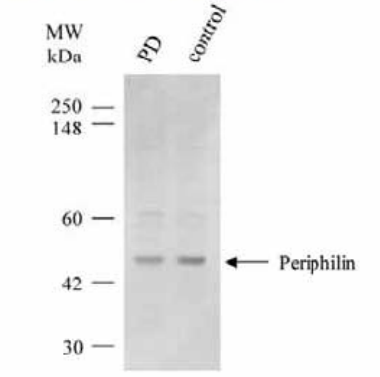
H P1 S1 P2 S2 S3 LP1 LS1 LP2 LS2
Periphilin Synphilin-1

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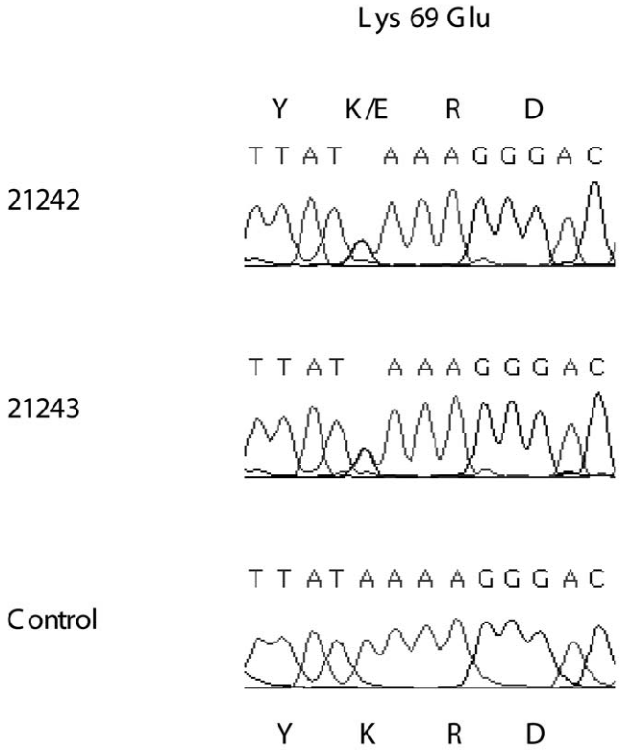
30
Periphilin

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                                    K69E
Hs_PPHLN1    MRDGFRRKSFYSSHYA-RERSPYKRDNTFFRESPVGRKDSPHSRSGSSVSSR
Mm_NP666174  MRDGFRRKSFYSSHYS-RDRSPHKRDAPFFRESPVGRKDSPHSRSGSSVSSR
Rn_XP345866  MRDGFRRKSFYSSHYS-RDRSPHKRDAPFFRESPVGRKDSPHSRSGSSVSSR
Gg_XP416035  VRDGFRRKSFHPSHYM-RERSPHKRDSPFFRESPVSRRDSPHSRSGSSVSSR
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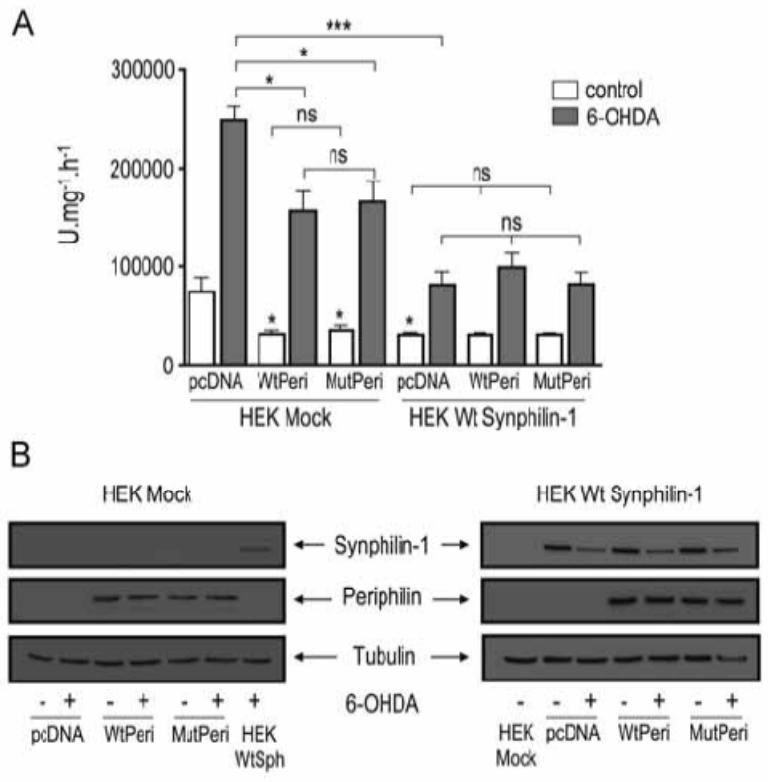

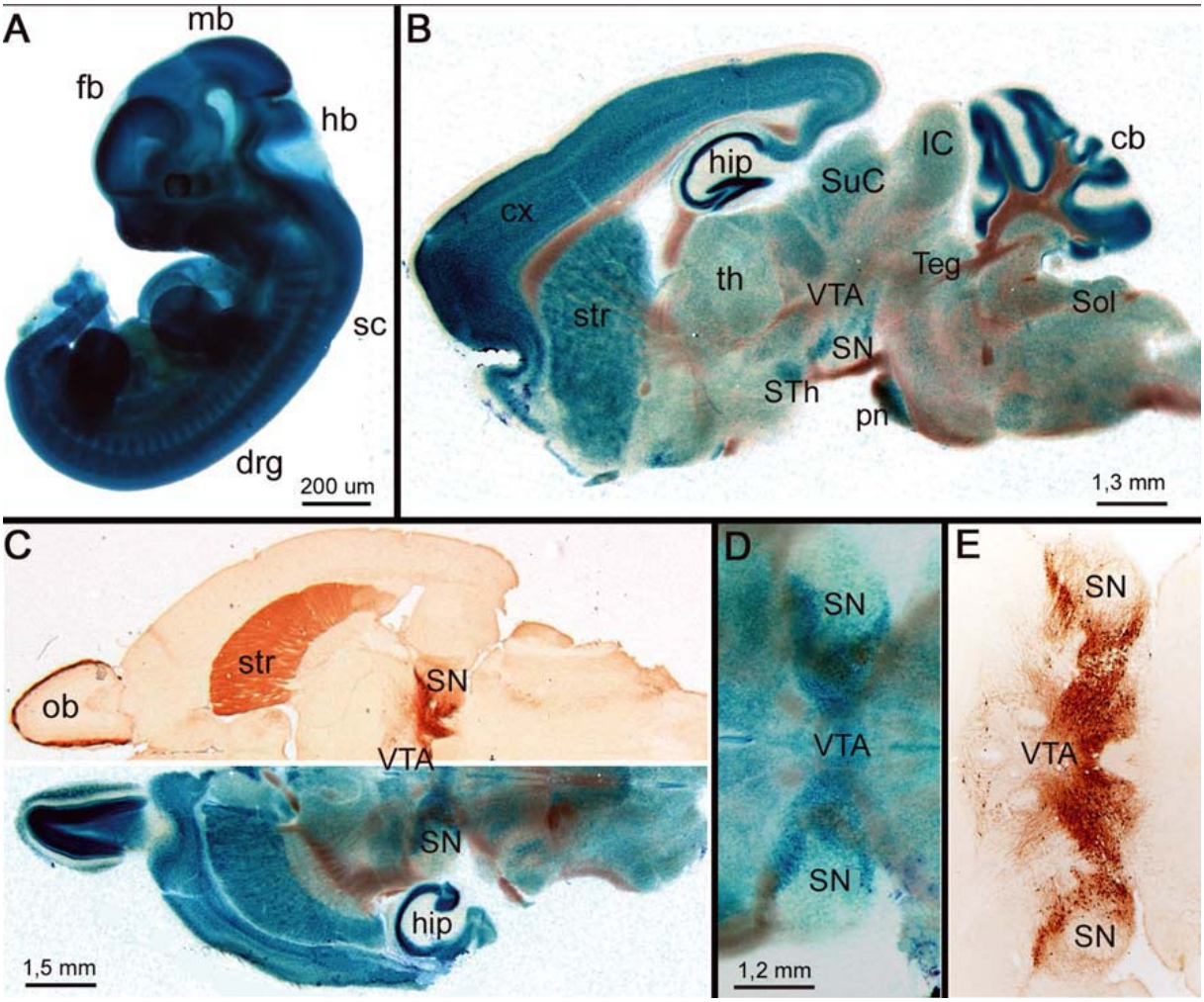
A
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1,3 mm
C
str
SN
ob
VTA
SN
hip
1,5 mm
D
SN
VTA
SN
1,2 mm
E
SN
VTA
SN

B.V.1) Les membres de la γ-secrétase (Articles 6, 7 et 8)

Article 6: "Study on the Putative Contribution of Caspases and the Proteasome to the Degradation of Aph-1a and Pen-2" Neuro-degenerative Diseases, 2007, 4(2-3), 156-63.

Article 7: "p53-dependent Control of Pen-2 Promoter Transcription by Presinilins and Evidence of a Feed-Back Control of Presinilin 1 and 2 Transactivation by Pen-2. Soumis.

Article 8: "p53-dependent Control of Cell Death by Nicastrin: Lack of Requirement for Presenilin-Dependent γ-secretase Complex. Soumis.

Dans le laboratoire du Dr Checler nous nous intéressons en particulier aux similitudes qui peuvent exister entre trois maladies neurodégénératives, à savoir la maladie de Parkinson, la maladie d'Alzheimer et les maladies à prions. Ces similitudes thématiques et techniques conduisent à d'étroites collaborations entre les différents membres du laboratoire. Dans ce cadre j'ai participé à des travaux ayant pour sujets d'études des protéines importantes dans le développement de la maladie d'Alzheimer.

Les marques histopathologiques caractéristiques de la maladie d'Alzheimer sont des dégénérescences neurofibrillaires, une mort neuronale massive et les plaques séniles. Ces plaques sont majoritairement constituées de peptide amyloïde agrégé que l'on nomme Aβ. Ce peptide est issu du clivage de son précurseur une protéine transmembranaire, la βAPP par deux activités enzymatiques distinctes, que l'on nomme β- et γ-secrétases. Les publications auxquelles j'ai participé abordent les régulations et les fonctions de trois des membres de ce complexe protéique γ-secrétase, qui est composé des préséniline 1 ou 2, de la nicastrine, d'une isoforme d'Aph-1 et de la protéine Pen-2 (Figure 43).

Dans ces différents travaux nous nous sommes particulièrement intéressés à la fonction de ces protéines dans les mécanismes d'apoptose et l'influence de p53 sur

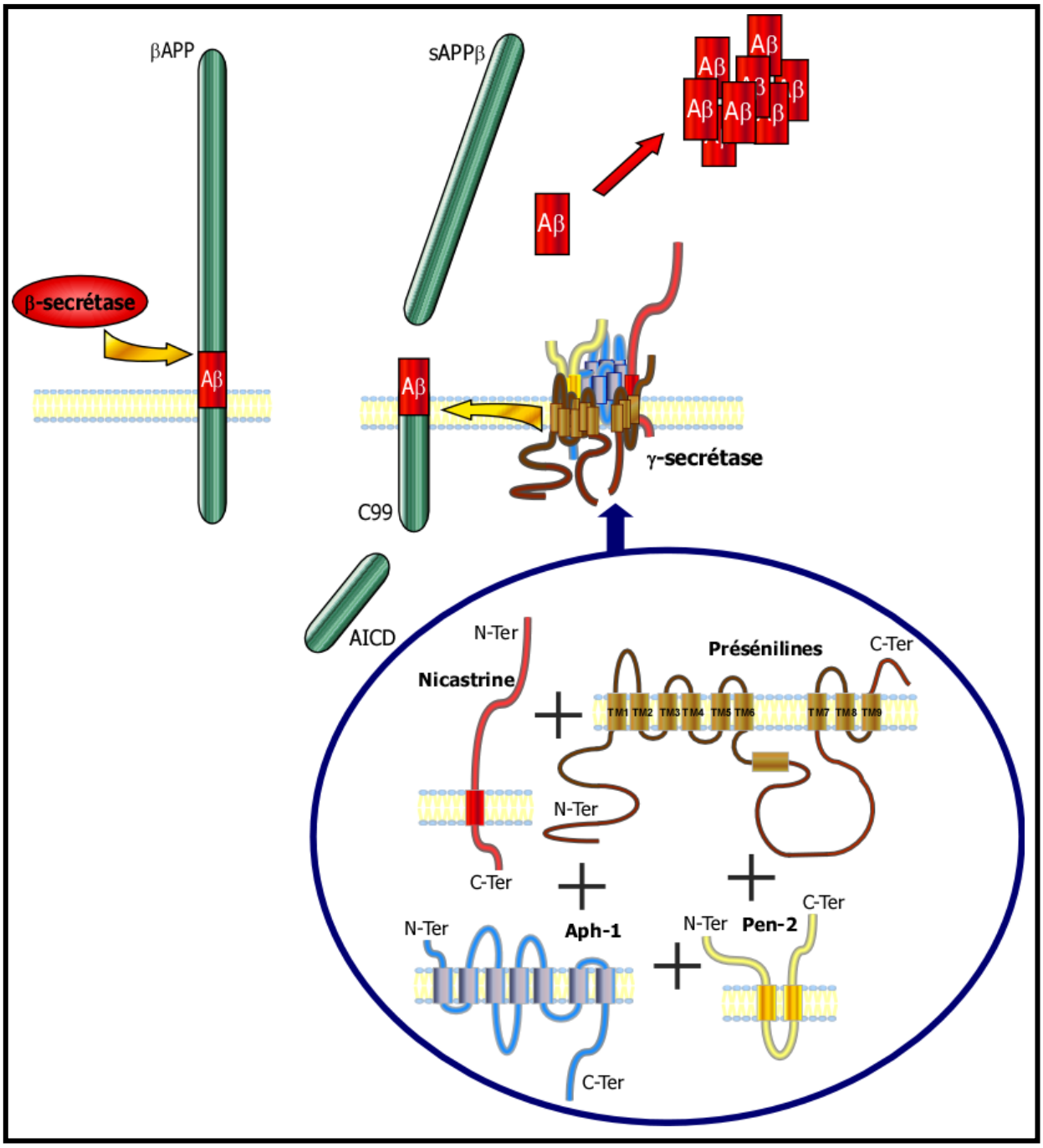

Figure 41 : Formation du complexe γ-secrétase et production du peptide amyloïde

La protéine transmembranaire βAPP va être clivée par la β-secrétase ce qui va libérer un fragment soluble sAPPβ et un fragment transmembranaire C99. Ce dernier va être clivé par l'activité γ-secrétase protée par le complexe multi-protéique formé de la nicastrine, d'Aph-1, de Pen-2 et d'une préséniline ce qui va produire le peptide amyloïde, Aβ et sa contrepartie intracellulaire, AICD.

ceux-ci, c'est d'ailleurs dans ce dernier aspect que se situe l'essentiel de ma collaboration. Nous avons démontré que la nicastrine, Aph-1 et Pen-2 réduisent la sensibilité des neurones à différents stimuli apoptotiques en inhibant en particulier l'activité des caspases effectrices. Les capacités régulatrices de ces trois protéines passent par la régulation de l'oncogène p53, en effet, l'expression de p53 est très fortement régulée par ces membres du complexe γ-secrétase. Cependant, les fonctions protectrices d'Aph-1 et de Pen-2 dépendent de l'intégrité du complexe tandis que celle de la nicastrine en est totalement indépendante. Dans ces travaux nous avons également pu mettre en évidence un circuit de régulation entre Pen-2, p53 et les préséniliines.

Article 6

Dunys J., Kawarai T., Giaime E., Wilk S., Herrant M., Auberger P., St George-Hyslop P., Alves da Costa C., Checler F.

"Study on the putative contribution of caspases and the proteasome to the degradation of Aph-1 and Pen-2."

Neurodegenerative Diseases. 2007; 4(2-3):156-63

Neuro–degenerative Diseases

Neurodegenerative Dis 133-T2
DOI: 10.1159/0000XXXXX

Published online: ■■■

Study on the Putative Contribution of Caspases and the Proteasome to the Degradation of Aph-1a and Pen-2

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Key Words
■■■ · ■■■

Abstract
The presenilin-dependent γ-secretase complex is mainly composed of four distinct proteins, namely presenilin 1 or presenilin 2, nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer (Pen-2). The mechanisms by which the complex is assembled, how its stochiometry is controlled and how its catalytic activity is regulated are poorly understood. Recent studies indicated that Aph-1 and Pen-2 undergo proteolysis by the proteasome. We have examined the susceptibility of endogenous and overexpressed Aph-1a and Pen-2 to proteolysis by endogenous and purified proteasome as well as by recombinant caspases. We show that endogenous Aph-1a and Pen-2 resist proteolysis by caspases and by the proteasome. Furthermore, we show that unexpected interference of proteasome inhibitors with the cmv promoter region driving expression of Aph-1a and Pen-2 led to artifactual enhancement of overexpressed Aph-1a and Pen-2-like immunoreactivities but that these proteins also resist to in vitro degradation by endogenous and purified proteasome.

Introduction

The amyloid β-peptides that accumulate in Alzheimer's disease-affected brains are generated by the sequential cleavages of the β-amyloid precursor protein by β-secretase and γ-secretase [1]. Because amyloid-β-peptide (Aβ) likely plays a key role in AD pathology [2], studies aimed at identifying γ-secretase and designing selective inhibitors have been numerous. There exists presenilin-independent [3–6] and presenilin-dependent [7, 8] γ-secretase activities. The latter has been characterized as a high molecular weight complex including at least presenilins 1 or 2 as well as three other proteins named nicastrin, Aph-1 (anterior pharynx defective) and Pen-2 (presenilin enhancer) [9, 10]. These proteins interact both physically and functionally and build the γ-secretase complex in a co-ordinated manner [11, 12].

Very few data are yet available concerning the catabolism of endogenous Aph-1 and Pen-2. Thus, Crystal et al. [13] only studied the fate of overexpressed Aph-1a and Pen-2. On the other hand, Bergman et al. [14] examined the degradation of Aph-1a or Pen-2 in presenilin-deficient blastocysts or in cells transiently transfected with PS1 cDNA. However, no study has examined the catabo-

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lism of endogenous Aph-1a and Pen-2 by cells displaying physiological contents of the various γ-secretase components. Here we demonstrate that, unlike previously documented, proteasomal degradation of overexpressed Aph-1a and Pen-2 are artifactual and that endogenous proteins also resist proteolysis by proteasome and caspases, in vitro and in vivo. Furthermore, we confirm and extent our previous data [15] indicating that Aph-1a and Pen-2 stability was drastically decreased in cells in which one member of the γ-secretase complex was lacking.

Materials and Methods

Inhibitors and Cells

Commercial protease inhibitors, Z-IE(Ot-Bu)A-Leucinal and Z-L-Leucinal and cell lines were those described previously [15].

cDNA Constructions and Mutagenesis

Aph-1a, Aph-1b and Pen-2 cDNAs were cloned in pcDNA4-myc-His vector (Invitrogen). Aph-1aD64A and Aph-1bD64A mutations were engineered using 'QuickChange Site-Directed Mutagenesis Kit' (Stratagene) according to the manufacturer's recommendations with the following set of primers: for Aph-1b forward primer: 5′-GTC-AAT-AAT-GAC-AAC-AAA-GCA-GGA-CCA-ACA-CAG-3′ and backward primer: 5′-GTC-TGT-TGG-TCC-TGC-TTT-GTT-GTC-AAT-AAT-GAC-3′; for Aph-1a forward primer: 5′-GTG-ACC-GAC-CGG-TCA-GCA-GCC-CGG-CTC-CAG-TAC-3′ and backward primer 5′-GTA-CTG-GAG-CCG-GGC-TGC-TGA-CCG-GTC-GGT-CAC-3′. Mutations were confirmed by sequencing.

In vitro Transcriptional/Translation of Wild-Type and Mutated Aph-1a/b and Cleavage by Caspases-3, -6 and -7 in a Cell-Free System

Wild-type Aph-1a, Aph-1b, mutated Aph-1aD64A and Aph-1bD64A and synphilin-1 were transcribed and translated using the Promega TNT coupled reticulocyte lysate system in the presence of ^{35}S-methionine (ICN), with 25 ng of recombinant caspase-3, -6 and -7 (Sigma) for 8 h at 37°C as extensively described [16]. Proteins were then electrophoresed on polyacrylamide gels and autoradiographed using Amersham hyperfilms.

Western Blot Analysis and Antibodies

Immunoreactivities were analyzed by separation of equal amounts of proteins on Tris-glycine gels for Aph-1a, actin and tubulin, or on Tris-tricine gels for Pen-2. The proteins were transferred to nitrocellulose membranes (Hybond-C Amersham Biosciences) blocked with nonfat milk and probed overnight with the following primary antibodies: anti-myc 9E10 (AVENTIS), anti-Pen-2 PNT2 (a gift from Dr. Xuaxi Xu, New York, N.Y., USA), anti-Aph-1a H2D2 (Calbiochem, San Diego, Calif., USA), anti-actin and anti-tubulin human/mouse monoclonal antibodies (Sigma). Immunological complexes were revealed using an electrochemoluminescence method as described [17].

Cellular and Purified Proteasomal Activity Measurements

For measurements of cellular proteasomal activity, TSM1 (telencephalon-specific mu) cells and fibroblasts were cultured in the presence of proteasome inhibitors then harvested and lysed by hypotonic shock with Tris-HCl 10 mM pH 7.5. Homogenate proteins were incubated under agitation for 1 h at 37°C either with Z-Leu-Leucinal, Z-IE(Ot-Bu)A-Leucinal or lactacystin as described [15]. Activity was measured using a fluorogenic specific substrate of the proteasomal chymotrypsin-like activity (Z-Gly-Gly-Leu-7AMC; Affiniti, Mamhead, UK) as extensively described [15]. Purified bovine pituitary 20S proteasome was obtained as described [18] and assayed with either its fluorimetric substrate or myc-tagged Aph-1 and Pen-2 prepared from TSM1 overexpressing neurons as described [15].

Statistical Analysis

Statistical analysis was performed with Prism Software (Graphpad Software, San Diego, Calif., USA) using the Neuman-Keuls multiple comparison test for one-way analysis of variance.

Results

Myc-Tagged Aph-1 and Pen-2 Are Not Degraded by Serine, Thiol and Aspartyl Proteases in TSM1 Cells and Resist Proteolysis by Recombinant Caspase-3, -6 and -7

We have examined whether inhibitors of serine, thiol and aspartyl proteases could alter the myc-tagged Aph-1a or Pen-2 immunoreactivities in stably transfected TSM1 cells. Figure 1A shows that Aph-1a-myc and Pen-2-myc immunoreactivities were not modified by AEBSF (4-2aminoethylbenzene-sulfonyl fluoride) and pepstatin. A faint protection of Pen-2 expression, but not Aph-1a was observed after treatment with the cysteine-protease inhibitor E64 (L-transepoxysuccinyleucilamino(n-guanido) butane) (fig. 1a). Interestingly, this faint protection was also observed with Ac-DEVD-al, an inhibitor of caspases including caspase-3 that belongs to the cysteine-protease group. This prompted us to examine whether recombinant caspase-3, -6 and -7 could account for the Ac-DEVD-al-sensitive Pen-2 protection although Pen-2 does not harbor any canonical targeted sequences for these caspases. Indeed, Pen-2 resisted degradation by recombinant caspase-3, -6 and -7 (not shown). Unlike Pen-2, Aph-1a and Aph-1b display a putative sequence that could behave as a caspase target (fig. 2a). We therefore mutate the putative caspase-3 cleavage site and examined first whether Aph-1a/b could be proteolysed by recombinant caspases and, second if their mutated counterparts could resist such possible degradation. Figure 2b clearly shows that both parent and mutant proteins resist degradation by recombinant caspases while synphilin-1 (fig. 2c)

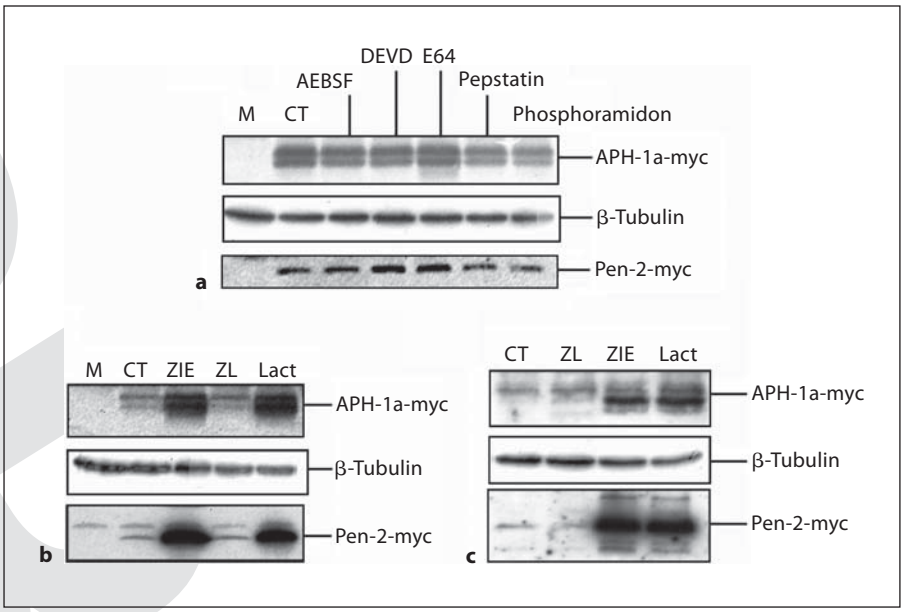


Fig. 1. Aph-1a-myc and Pen-2-myc immunoreactivities are not increased after treatment with various protease inhibitors but are enhanced by proteasome inhibitors. **a**, **b** TSM-1 neurons transfected for either Aph-1a-myc or Pen-2-myc were treated for 16 h without (CT) or with the indicated inhibitor at the following concentrations: AEBSF (100 μM), Ac-DEVD-al (DEVD, 100 μM), E64 (100 μM), pepstatin (10 μM), phosphoramidon (10 μM), Z-IE(Ot-Bu)A-Leucinal (ZIE, 10 μM), Z-leucinal (ZL, 10 μM), and lactacystin (Lact, 10 μM) then Aph-1a-myc- and Pen-2-myc–like immunoreactivities were monitored as described in 'Materials and Methods'. **c** HEK293 stably transfected cells were also treated for 16 h without (CT) or with 10 μM of Z-IE(Ot-Bu)A-Leucinal, Z-leucinal or lactacystin. β-Tubulin-like immunoreactivity served as protein loading control.

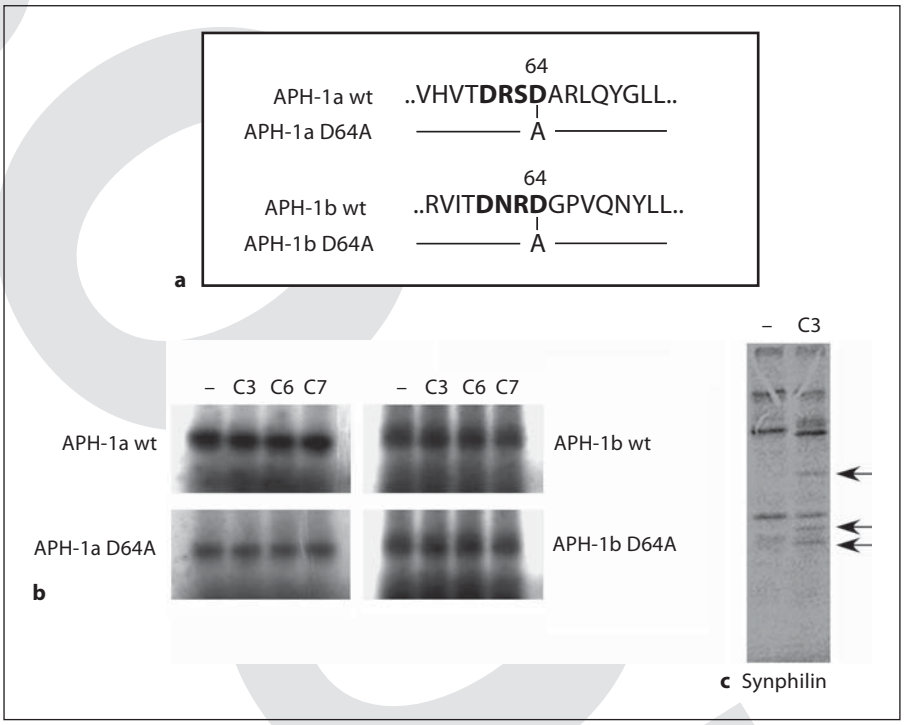


Fig. 2. Aph-1a or -b are not cleaved by recombinant caspases. **a** Aph-1a and Aph-1b present both a putative caspase-3 cleavage sites (in bold). Aph-1a D64A and Aph-1b D64A were mutated on the second aspartate of the putative cleavage site. **b** Wild-type Aph-1a/b or mutant Aph-1a/b D64A were transcribed and translated in vitro with ^{35}S-methionine and incubated for 8 h at 37°C with purified recombinant caspase-3, -6 and -7 (C3, C6, C7). **c** As in **b** with synphilin-1 incubated with recombinant caspase-3. Arrows indicate cleavage products. The reaction mixes were analyzed by SDS-PAGE as described in 'Materials and Methods'.

was readily cleaved by recombinant caspase-3 in a Z-VAD-al-sensitive manner [16] as previously described. Overall, the above data indicated that Aph-1a and Pen-2 were not degraded by serine, acidic and thiol proteases and resist proteolysis by caspase-3, -6 and -7.

Overexpressed and Endogenous Aph-1a and Pen-2 Display Distinct Susceptibility to Proteasome Inhibitors

The only inhibitors that drastically increased the immunoreactivities of overexpressed Aph-1a-myc and Pen-2-myc in TSM1-transfected cells were lactacystin and ZIE-(Ot-Bu)A-Leucinal (ZIE) (fig. 1b, c). Lactacystin is a

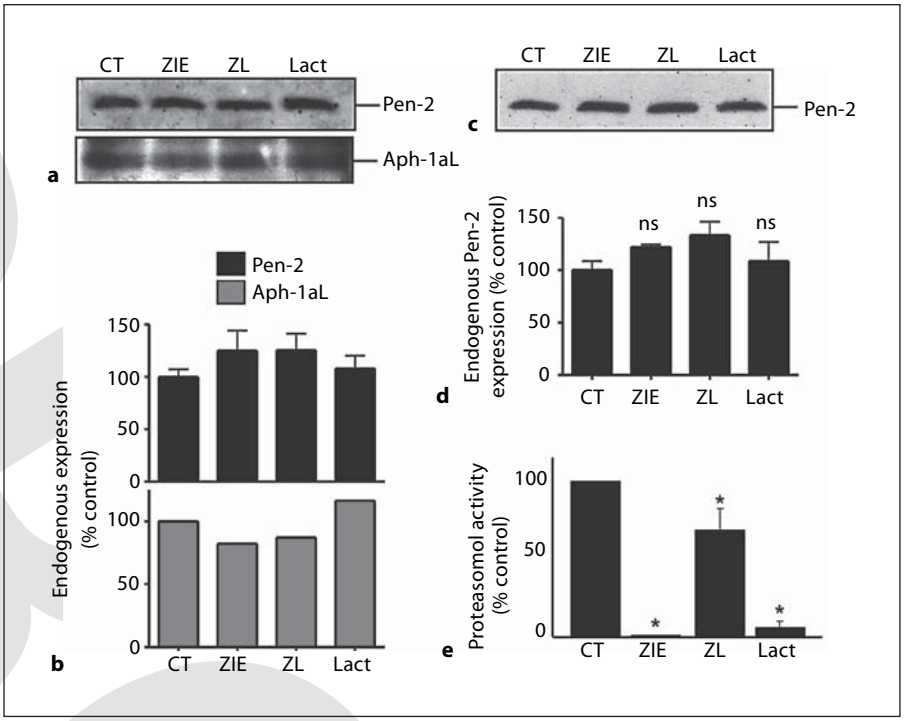


Fig. 3. Endogenous Pen-2 and Aph-1aL-like immunoreactivities are not increased by treatment with proteasome inhibitors in HEK-293 cells nor in primary cultured neurons. Mock-transfected HEK-293 cells and primary cultured neurons were treated for 16 and 24 h, respectively, with a 10-μM concentration of the indicated inhibitor then expression of endogenous Pen-2 (**a**, **c**) or Aph-1aL (**a**) were analyzed by Western blot using anti-Pen-2 (PNT2) and anti-Aph-1aL (H2D2) polyclonal antibodies as described in 'Materials and Methods'. Z-GGL-7AMC-hydrolyzing activity (**e**) was monitored as described in 'Materials and Methods'. Bars in **b** and **d** indicate the means of two or three (± SEM) independent experiments. * $p < 0.001$ compared with untreated cells; ns = nonsignificant.

rather specific inhibitor of the proteasome [19] while ZIE is a mixed inhibitor that targets not only the proteasome but also calpains and cathepsin B [20]. The putative contribution of the two latter enzymes was ruled out by the use of ZL-Leucinal (ZL) that inhibits calpains and cathepsin B but not the proteasome (fig. 1b). The same protective effect of lactacystin and ZIE was observed in Aph-1a- and Pen-2-transfected HEK293 cells (fig. 1c). Strikingly, endogenous Aph-1aL and Pen-2 expressions were not enhanced by ZIE and lactacystin in HEK293 cells (fig. 3a, b) and in primary cultured neurons (fig. 3c, d) in conditions where all proteasomal activity was indeed blocked (fig. 3e).

These clearly distinct effects of proteasome inhibitors on endogenous and transfected Aph-1a and Pen-2 could have two explanations. Either levels of expression of transfected Aph-1a and Pen-2 were high, yielding an overload of proteins that would be misfolded and cleared off by the proteasome machinery or, alternatively, lactacystin could aspecifically interact with the cmv promoter driving the expression of the transfected proteins, as had been shown for prion and α-synuclein [21–23], thereby leading to cmv-associated artifactual increase in the Aph-1a and Pen-2 immunoreactivities. These two problems could be overcome by an in vitro approach consisting in the study of endogenous and overexpressed Aph-1a and Pen-2 degradation by either cell homogenates displaying endogenous proteasome or by purified 20S proteasome. In cell homogenates, the artifactual interference of the cmv promoter with proteasome inhibitors would no longer stand. Any protection by lactacystin would indeed result from the blockade of the degradation of overexpressed proteins by endogenous proteasome. On the other hand, any putative misfolded Aph-1a- and Pen-2-myc proteins resulting from their overload would be avidly degraded by purified proteasome that is mainly constituted by the 20S counterpart that degrades preferentially natively unfolded or misfolded proteins [24]. Our study clearly showed that proteasome inhibitors did not protect overexpressed Aph-1a and Pen-2 from degradation by cell homogenates, i.e. by endogenous proteasome (fig. 4a, b) while these inhibitors fully block the proteasome activity (fig. 4c). Furthermore, purified highly active 20S proteasome (fig. 4f) did not degrade endogenous and overexpressed Aph-1a and Pen-2 (fig. 4d, e). Overall our data show that proteasome did not degrade Aph-1a and Pen-2.

Invalidation of Members of the γ-Secretase Complex Destabilize Endogenous Aph-1aL and Pen-2

Aph-1aL and Pen-2 physically and functionally interact with presenilins (PS) and nicastrin (NCT) to yield a high-molecular-weight γ-secretase complex [25–27]. We

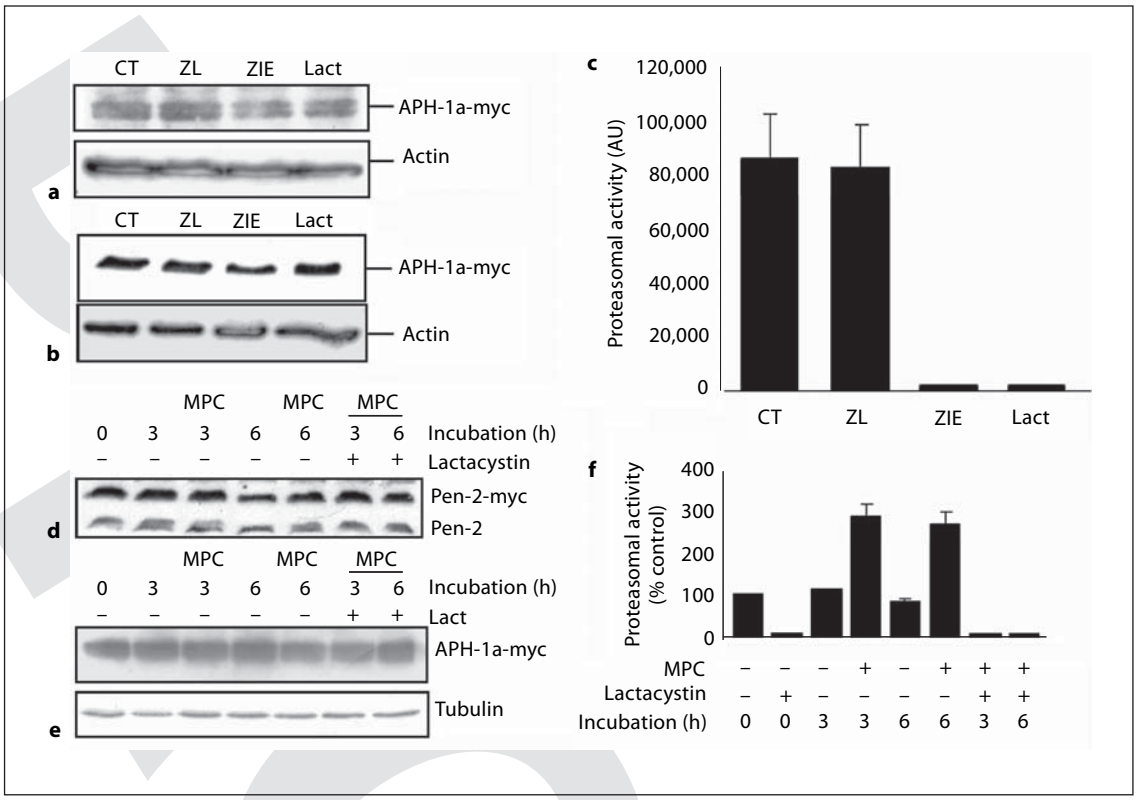
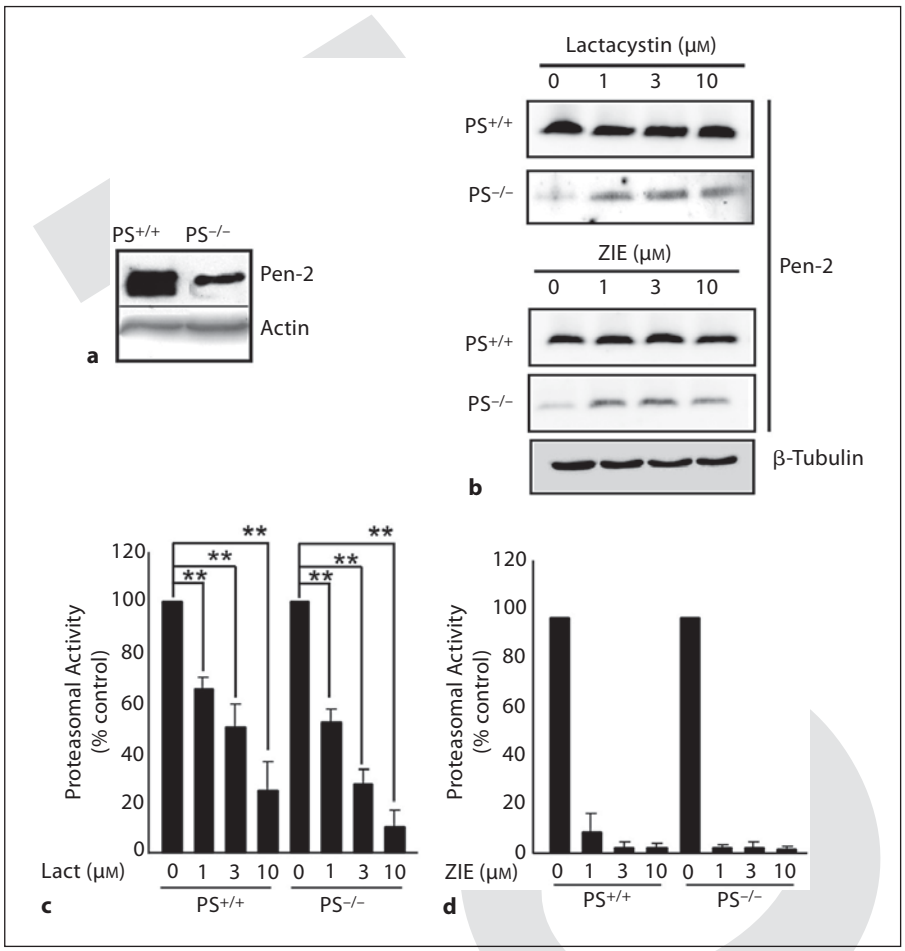


Fig. 4. Effect of proteasome inhibitors on the degradation of Aph-1-myc and Pen-2-myc-like by endogenous and purified 20S proteasome in TSM1 homogenates. **a**, **b** Homogenates of stably transfected TSM-1 neurons overexpressing myc-tagged Aph-1a (**a**) or Pen-2 (**b**) were incubated at 37°C for 1 h without (CT) or with a 10-μM concentration of the indicated inhibitor. myc-tagged proteins expressions were monitored by Western blot as described in 'Materials and Methods'. Actin-like immunoreactivity was used as control of protein loading. **c** Effect of proteasome inhibitors on the chymotrypsin-like activity of proteasome was monitored in cell homogenates as described in 'Materials and Methods'. Bars indicate means ± SEM of three independent determinations. ZIE = Z-IE(Ot-Bu)A-Leucinal; ZL = Z-Leu-Leucinal; Lact = lactacystin. Homogenates of stably transfected TSM-1 neurons overexpressing myc-tagged Pen-2 (**d**) or Aph-1a (**e**) were incubated with or without purified 20S proteasome (MPC) for the indicated time periods in the presence (+) or absence (–) of lactacystin (10 μM). We used polyclonal anti-Pen-2 (PNT2 antibody that recognizes both Pen-2 and myc-tagged Pen-2) or anti-myc antibody 9E10 to monitor, respectively, endogenous and myc-tagged Pen-2- (**d**) or Aph-1a immunoreactivities (**e**). β-Tubulin was used as control of loaded proteins. **f** Samples were analyzed for their proteasomal activity as described in 'Materials and Methods' by means of the fluorimetric substrate of the proteasomal chymotrypsin-like activity (Z-Gly-Gly-Leu-7AMC). Bars indicate the means ± SEM of three independent experiments.

have examined the fate of endogenous Pen-2 in cells devoid of presenilins and nicastrin. Clearly, Pen-2 expression was drastically lower in $PS^{-/-}$ and $Nct^{-/-}$ than in wild-type fibroblasts (fig. 5a, 6b, c) while Pen-2 expression was not affected by β-amyloid precursor protein (βAPP) and amyloid precursor-like protein 2 (APLP2) deficiencies. Here again, we confirmed that proteasome inhibitors did not modify endogenous Pen-2 expression in various wild-type fibroblast cell lines (fig. 5b, 6a, b) in conditions where fibroblastic proteasomal activity was fully inhibited by lactacystin (fig. 5c) and ZIE (fig. 5d). Conversely, proteasome inhibitors displayed faint and dose-independent protection of endogenous Pen-2 in $PS^{-/-}$ and $Nct^{-/-}$ fibroblasts (fig. 5b, 6c). However, purified 20S proteasome did not degrade endogenous Pen-2 prepared from $PS^{-/-}$ fibroblasts (not shown).

Discussion

The occurrence of two presenilins 1 and 2, three Aph-1a, b and c homologs [10, 28] and two splice variants of Aph-1a (Aph-1aL and Aph-1aS) [10, 29], leads to various theoretical combinations of the above proteins. The oc-



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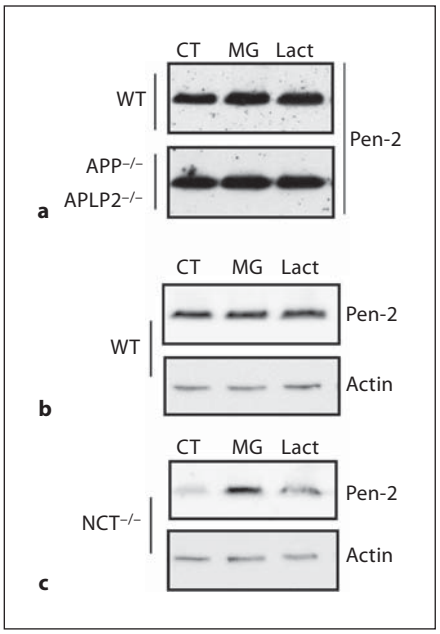

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Fig. 5. Influence of presenilins on endogenous Pen-2 levels and sensitivity to proteasome inhibitors. **a** Endogenous Pen-2-like immunoreactivity in fibroblasts wild-type ($PS^{+/+}$) or knock-out for presenilins ($PS^{-/-}$) was analyzed by Western blot using anti-Pen-2 (PNT2) as described in 'Materials and Methods'. **b–d** $PS^{+/+}$ and $PS^{-/-}$ fibroblasts were treated with the indicated concentrations of lactacystin (**b**, **c**) or ZIE (**b**, **d**) then Pen-2-like immunoreactivity (**b**) or proteasomal activity (**c**, **d**) were measured by Western blot or fluorimetric assay as described in 'Materials and Methods'. Bars are the means of three (**c**) or two (**d**) independent experiments. ** $p < 0.001$ compared to untreated cells.

Fig. 6. Influence of nicastrin, βAPP and APLP2 deficiency on endogenous Pen-2 and sensitivity to proteasome inhibitors. βAPP/APLP2 ($APP^{-/-}APLP2^{-/-}$)-deficient fibroblasts (**a**) or presenilin-expressing (WT) (**b**) and nicastrin ($NCT^{-/-}$)-deficient fibroblasts (**c**) were treated with a 10-μM concentration of the indicated inhibitor then endogenous Pen-2-like immunoreactivities was monitored by Western blot.

currence of such distinct complexes has now been confirmed [30, 31] and it is generally admitted that the term 'presenilin-dependent γ-secretase activity' refers to a series of 'γ-secretase complexes'. Obviously, this adds another degree of complexity for the understanding of the biology and specific roles of the various γ-secretase complexes. Several major questions remain and particularly whether each of these γ-secretase complexes displays a specific function and targets selective substrates. The mechanisms by which levels of a given complex are regulated remain also a mystery but it is likely that endogenous concentrations of the complexes could be directly controlled by proteolytic events.

Very few studies on the degradation of endogenous Aph-1 and Pen-2 have been documented. Crystal et al. [13] reported on the involvement of the proteasome in the catabolism of overexpressed Aph-1a and Pen-2. We found discrepant data when examining the effect of proteasome inhibitors on endogenous and overexpressed Aph-1a and Pen-2 levels. Thus, lactacystin and ZIE enhanced overexpressed Aph-1a and Pen-2-like immunoreactivities in HEK293 cells and TSM1 neurons while they do not affect

endogenous levels of these proteins in HEK293 cells and primary cultured neurons. We demonstrated that both endogenous and overexpressed Aph-1a and Pen-2 prepared from cell homogenates remained insensitive to proteasome inhibitors in vitro, and were not degraded by purified 20S proteasome. Therefore, we conclude that Aph-1a and Pen-2 do not behave as substrate of the proteasome [15] and that the protection of overexpressed Aph-1a and Pen-2 immunoreactivities by lactacystin and ZIE was artifactually due to the interference of these inhibitors with the cmv promoter driving the expression of Aph-1a and Pen-2 in pcDNA3 as has been shown for other proteins [21–23].

The thiol protease inhibitor E64 slightly enhanced Pen-2-like immunoreactivity in TSM1 neurons (fig. 1) and HEK293 cells [15]. Interestingly, Ac-DEVD-al, an inhibitor of thiol proteases called caspases mimicked the E64-induced protection. As several studies indicated that other members of the γ-secretase complex, namely presenilins undergo caspase-3 cleavages that modulate their ability to control cell death for reviews see [32, #2242; 33, #10575], we examined whether Pen-2 and Aph-1a behaved as substrates of recombinant caspases, in vitro. Clearly, Aph-1a and Pen-2 also resisted proteolysis by recombinant caspases.

We confirmed that presenilin deficiency destabilizes Pen-2. We extend these data by showing that nicastrin deficiency also drastically reduces the levels of endogenous Pen-2 while Pen-2 levels were not affected by combined βAPP and APLP2 deficiencies. Therefore, one could conclude that Aph-1a and Pen-2 are metabolically stable when inserted within the γ-secretase complex and rapidly broken down when occurring outside of this structure. However, our data indicate that 20S proteasome was apparently not responsible for Pen-2 degradation in presenilin- and nicastrin-deficient fibroblasts.

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Article 7

Dunys J., Sevalle J., Giaime E., Vitek M., Renbaum P., Levy-Lahad E., Zhang Y-W., Xu H., Alves da Costa C., Checler F.

"p53-dependent control of Pen-2 promoter transcription by presenilins and evidence of a feed-back control of presenilin 1 and 2 transactivation by Pen-2."

En révision

p53-DEPENDENT CONTROL OF PEN-2 PROMOTER TRANSCRIPTION BY PRESENILINS AND EVIDENCE OF A FEED-BACK CONTROL OF PRESENILIN 1 AND 2 TRANSACTIVATION BY PEN-2

Abbreviated title: p53 regulates the transactivation of Pen-2 promoter

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Abbreviations:

AD, Alzheimer's disease; Aph-1, anterior pharynx defective-1; Pen-2, presenilin enhancer-2; NCT, nicastrin; APP, amyloid precursor protein; PS, presenilin; PFTα, pifithrin-α; MEF, mouse embryonic fibroblasts; FAD, familial Alzheimer's disease; AICD, βAPP intracellular domain; ARF, alternative reading frame; TSM, telencephalon specific murine.

Abstract

Alzheimer's disease is a neurodegenerative disorder characterized by neuronal loss, neurofibrillary tangles and senile plaques. The latter lesions are mainly due to the accumulation of hydrophobic peptides referred to as amyloid β-peptides (Aβ). Aβ peptides are liberated by γ-secretases, a generic term for a high molecular weight complex including presenilins, Pen-2, Aph-1 and nicastrin. Previous reports indicated close biophysical and functional interactions between these proteins. Thus, depletion of each of these proteins disrupts the ability of the complex to properly assemble into a functional protease, thereby reducing Aβ production. Here we describe another level of regulation of this multimeric protease. The depletion of both presenilins drastically reduces the transactivation of the Pen-2 promoter. Furthermore, PS1 over-expression lowers Pen-2 promoter transactivation, a phenotype abolished by a double mutation known to prevent PS-dependent γ-secretase activity. Pen-2 promoter transactivation was decreased by βAPP depletion and increased by the APP intracellular domain (AICD). Interestingly, presenilin-2 over-expression highly increases Pen-2 promoter transactivation. The opposite effect triggered by presenilin-1 and presenilin-2 was reminiscent of our previous study showing that these two proteins trigger antagonistic effects on p53. Therefore, we examined the putative contribution of p53 on Pen-2 transactivation. Pen-2 promoter transactivation and expression are sensitive to the p53 inhibitor pifithrin-α, are drastically reduced in $p53^{-/-}$ fibroblasts and can be rescued by p53 complementation. Furthermore, we show that Pen-2 modulates presenilin-1 and presenilin-2 promoter transactivation in a p53-dependent manner. Overall, our study describes a p53-dependent cross-talk between presenilins and Pen-2 controlling their respective levels of promoter transactivation.

Introduction

Alzheimer disease (AD) is characterized by the extracellular cortical deposition of senile plaques, the main component of which is a set of poorly soluble peptides named amyloid β-peptides. That these peptides account for the etiology of the disease still awaits definite proof while genetic clues support the assumption that Aβ peptides at least contribute to the genesis of Alzheimer's disease. Thus, mutations on distinct proteins, namely the β-amyloid precursor protein (βAPP) and presenilin (PS) 1 and PS2, are both responsible for early onset and aggressive forms of neurodegeneration (Tanzi, 1999; Selkoe, 2001). A common influence of these mutations is to perturb the processing of βAPP, yielding modified levels of Aβ-like peptides (Checler, 1995). These considerations support the huge effort to identify the enzymes responsible for the genesis of Aβ since theoretically, any pharmacological compound able to interfere with Aβ production could be seen as a putative tool to slow down or arrest AD pathology and/or progression.

Aβ derives from the proteolytic attack of its transmembrane precursor βAPP by β- and γ-secretases that liberate the N- and C-termini, respectively (Checler, 1995). γ-secretase refers to both PS-dependent (De Strooper et al., 1998) and PS-independent activities (Armogida et al., 2001; Wilson et al., 2002; Wilson et al., 2003; Beglopoulos et al., 2004; Lai et al., 2006). The former has been characterized as a high molecular weight complex composed of PS1 or PS2, Pen-2, Aph-1 and nicastrin (Goutte, 2000; Herreman et al., 2000; Yu et al., 2000; Zhang et al., 2000; Francis et al., 2002; Edbauer et al., 2003; Takasugi et al., 2003). Each of the above proteins has been described as a limiting factor to build up a biologically active γ-secretase complex. Thus, the absence of any of these proteins disrupts the catalytic function of the complex (Herreman et al., 2000; Zhang et al., 2000; Li et al., 2003; Ma et al., 2005) while a catalytically active entity can be reconstituted by stochiometric addition of the four proteins (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). Cell biology approaches allowed better understanding of the interdependency between the members of the complex.

Thus, Aph-1 and nicastrin initially form a stabilized sub-complex (Hu and Fortini, 2003; La Voie et al., 2003) that then incorporates either PS1 or PS2 (Lee et al., 2002). The final maturation step involves the addition of Pen-2 that appears necessary for PS to undergo endoproteolytic maturation (Luo et al., 2003; Takasugi et al., 2003).

The corollary of such a stringent contribution of each of these proteins to the γ-secretase complex is that an important effort should be done for a better understanding of the regulation of their expression but very little is known concerning subunit expression. Studies of the post-transcriptional regulation of the levels of the various members of the complex concerned their catabolic fate. Clearly, proteins are stabilized when they are included in the complex while the lack of one protein apparently drastically accelerates the catabolism of the others. Thus, lowering Aph-1 expression by a siRNA approach clearly reduces PS expression (Lee et al., 2002) while Pen-2 expression is drastically lowered in PS- and nicastrin-deficient fibroblasts (Zhang et al., 2005; Dunys et al., 2006).

Very little is known concerning the transcriptional regulation of the members of the γ-secretase complex. PS1 gene transcription is activated by CREB (Mitsuda et al., 2001) and down regulated by the tumor suppressor p53 (Roperch et al., 1998; Pastorcic and Das, 2000) while PS2 is increased by early growth response gene-1 (Renbaum et al., 2003). Pen-2 transcription is up-regulated by CREB (Wang et al., 2006b). Here we establish that presenilins regulate p53-dependent activation of the Pen-2 promoter. Furthermore, we establish a feed back control by which Pen-2 retro-controls the expression of PS1 and PS2 in a p53-dependent manner.

Experimental Procedures:

Cell culture and tranfections

Stably transfected HEK293 cells expressing wild-type (wt)-PS1, D257A/D385A- PS1 or wt-PS2 were obtained after transfection of 3µg of cDNA using Lipofectamine reagent (Invitrogen) according to manufacturer's recommendations and selection of positive transfectants by Western Blot as described below. Fibroblasts were transfected with Lipofectamine 2000 reagent (Invitrogen) or JetPEI reagent (Polyplus-transfections) according to manufacturers recommendations. In some experiments, fibroblasts were transfected by means of the mouse embryonic fibroblasts NucleofectorTM kit (Amaxa Biosystems, Koeln, Germany) as described (Alves da Costa et al., 2006). Mouse Embryonic fibroblasts (MEF) depleted of both PS1 and PS2 ($PS^{-/-}$) or of βAPP ($APP^{-/-}$) were cultured as previously described (Dunys et al., 2006). MEF devoid of $p19^{Arf-/-}$ or of both $p19^{Arf-/-}$ and $p53^{-/-}$ were cultured as previously described (Dunys et al., 2007). Telencephalon specific murine cells (TSM-1) over-expressing myc-tagged Pen-2 were obtained and cultured as previously described (Dunys et al., 2006).

Site-directed mutagenesis.

PS1 construct in which both aspartates D285 and D357 were replaced by an alanine residue (DD-PS1) was obtained by oligonucleotide-directed mutagenesis from human wild-type PS1 cDNA by means of a QuikChange site-directed mutagenesis kit (Stratagene,La Jolla, CA). Mutagenesis was performed according to the manufacturer's conditions using first, the set of primers 5'-GGC TGT GAT TTC AGT ATA TGC TTT AGT GGC TGT TTT GTG TCC G-3' and 5'-CGG ACA CAA AAC AGC CAC TAA AGC ATA TAC TGA AAT CAC AGC C-3' (Cybergene, Saint-Malo, France) containing the D257A mutation. Then, this mutant construct was used to produce the double mutant using the set of primers 5'-CTT GGA TTG GGA GCT TTC ATT TTC TAC AGT GTT CTG G-3' and 5'-CCA GAA CAC TGT AGA AAA

TGA AAG CTC CCA ATC CAA G-3' containing the D385A mutation. Final cDNA constructs were entirely sequenced to verify mutations.

Western blot analyses and antibodies

Cells were gently scraped with PBS-EDTA 5mM, pelleted by centrifugation, and then lysed in 50-100µl of 25mM HEPES, pH7,5 containing a cocktail of protease inhibitors (Roche Molecular Biochemicals). Equal amounts of protein were separated on SDS-PAGE gels containing 8% to 12% of acrylamide (Euromedex) for analysis of βAPP, PS1 and PS2 or on 16.5% acrylamide Tris-Tricine gels for PEN-2. Proteins were then wet-transferred to Hybond C membranes (GE HealthCare). Membranes were then blocked with non-fat milk and incubated over-night at 4°C with the following primary antibodies: anti-Pen-2 (PNT2, rabbit polyclonal, Calbiochem), anti-actin (mouse monoclonal, Sigma-Aldrich), anti-PS1-Nter (rabbit polyclonal, a gift from Dr Thinakaran), anti-PS2-Cter 2192 (rabbit polyclonal, Cell Signaling) and anti-βAPP 22C11 (mouse monoclonal, Boehringer, Ingelheim). Immunological complexes were revealed by enhanced electrochemiluminescence (Roche Molecular Biochemicals) with either anti-rabbit or anti-mouse IgG antibodies coupled to peroxidase (Jackson Immunoresearch) antibodies.

Real-time quantitative polymerase chain reactions:

RNAs from $p19^{Arf-/-}$ or $p19^{Arf-/-}p53^{-/-}$ fibroblasts were extracted by means of the Rneasy kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations, then treated with DNAse I then 4µg of total RNA were reverse transcribed as previously described (Alves da Costa et al., 2006). Real-time PCR was performed as extensively described (Dunys et al., 2007) with gene-specific primers for mouse Pen-2 and mouse actin to normalize mRNA concentrations.

Promoter activity measurements

The human Pen-2- (hpPen-2), murine PS1 (mPS1)- and human PS2 (hPD2)-promoters in frame with luciferase reporter gene have been previously described (Mitsuda et al., 1997; Renbaum et al., 2003; Wang et al., 2006b). Cells were cultivated in 12-cell plates until they reach 60%-70% confluence then they were co-transfected with 1.0μg of either hpPen-2-luciferase, mPS1-luciferase or hPS2-luciferase cDNA and 0.5μg of a β-galactosidase transfection vector (to normalize transfection efficiency) with or without 1.0μg of either pcDNA3, AICD C59 or p53 cDNA by means of the Lipofectamine 2000 reagent (Invitrogen) or the Amaxa Transfection System (Amaxa Biosystems, Koeln, Germany) according the manufacturers conditions. In a subset of experiments, twenty-four hours after transfection, stably transfected HEK293 cells expressing wt-PS1, wt-PS2 or mutated PS1were treated for 16 hours with or without the p53 inhibitor (Komarov et al., 1999) pifithrin-α (10μM). Luciferase and β-galactosidase activities were then analyzed according to manufacturer's recommendations (Promega).

Statistical analysis

Statistical analyses were performed using Prism Software (Graphpad Software, San Diego, CA) by the mean of the Neumans-Keuls Multiple comparison test.

Results

Presenilins regulate the transactivation of the Pen-2 promoter

As was consistently reported in previous studies (Bergman et al., 2004; Crystal et al., 2004; Dunys et al., 2006), Pen-2 expression is drastically reduced by the depletion of both PS1 and PS2 in fibroblasts (Fig.1A). We examined whether part of this phenotype could be accounted for by reduced Pen-2 gene transcription. Indeed, we establish that PS-depleted fibroblasts display a significant reduction of Pen-2 promoter transactivation (Fig.1B), indicating that reduced transcription of Pen-2 promoter could also contribute to the lowered levels of Pen-2 in these cells. Interestingly, Pen-2 expression in $PS^{-/-}$ fibroblasts could be enhanced by complementation with both PS1 and PS2 complementation (Fig.1C).

We examined whether PS1 and PS2 similarly influence Pen-2 promoter transactivation. Interestingly, PS1 and PS2 elicit an opposite effect. Thus, as was previously described, PS1 over-expression reduces PS2 expression (Fig. 2A) and that was accompanied by a reduction of Pen-2 promoter transactivation (Fig.2B). Conversely, PS2 over-expression reduced PS1 levels (Fig.2A) and drastically enhances Pen-2 promoter transactivation (Fig.2B).

Presenilin-dependent γ-secretase controls Pen-2 promoter transactivation via AICD

To investigate the role of presenilin-dependent γ-secretase activity in the regulation of Pen-2 promoter transcription, we analyzed the effect of the substitution of aspartate residues 257 and 385 of PS1 by alanines. This double mutation (DD-PS1) has been reported to abolish PS1-associated γ-secretase activity (Wolfe et al., 1999). Fig.3A shows that this double mutation abolishes the PS1-induced inhibition of Pen-2 promoter transactivation, suggesting a role of a γ-secretase-derived product in the control of Pen-2 transcriptional regulation. Two distinct lines of evidence suggest that this product could be AICD, the intracellular domain of βAPP that is released upon cleavage of βAPP by γ-secretase (Passer et al., 2000). First, the over-expression of AICD (C59 in Fig.3B) increases Pen-2 promoter transactivation (Fig.3C). Second,

fibroblasts devoid of βAPP display reduced Pen-2 expression (Fig. 4A,B) and lowered Pen-2 promoter activation (Fig. 4C). Overall, this suggests that the lack of endogenous AICD likely accounts for the reduced Pen-2 promoter activation and expression in βAPP-deficient fibroblasts.

p53 regulates Pen-2 promoter transactivation

We envisioned the possibility that PS-dependent and AICD-induced regulation of Pen-2 promoter transactivation could be mediated by p53 for two main reasons. First, the opposite effects of PS1 and PS2 on the transactivation of the Pen-2 promoter were strikingly similar to those triggered by these proteins on p53 expression, activity, promoter transactivation and mRNA levels (Alves da Costa et al., 2006). Second, we previously demonstrated that AICD could indeed act as a transcriptional regulator of p53 (Alves da Costa et al., 2006). Three lines of data support the view that p53 was responsible for the PS-associated regulation of Pen-2 promoter transactivation. First, PS1 and PS2-induced modification of Pen-2 promoter transactivation was fully abolished by pifithrin-α (Fig.5) a specific inhibitor of the p53 activity (Komarov et al., 1999). Second, Pen-2 expression (Fig.6A,B), promoter transactivation activity (Fig.6C) and quantitative real-time PCR measurements of Pen-2 mRNA levels (Fig.6D) were all drastically reduced by depletion of endogenous p53. Third, the transient transfection of p53 cDNA in $p19^{Arf-/-}p53^{-/-}$ fibroblasts restores Pen-2 expression (Fig.7A,B) and Pen-2 promoter activation (Fig.7C). Overall, our data demonstrate for the first time that p53 is a transcriptional regulator of Pen-2 promoter transactivation and indicate that PS could control Pen-2 transcription via an AICD-mediated control of this oncogene.

p53-dependent modulation of PS promoter transactivation by Pen-2.

Several studies indicated that PS1 expression was down regulated by p53 (Roperch et al., 1998; Pastorcic and Das, 2000). Furthermore, we recently showed that Pen-2 could lower p53 expression and promoter transactivation (Dunys et al., 2007).

This prompted us to examine whether a feed back mechanism by which Pen-2 would modulate PS1 or PS2 promoter transactivation via its control of p53 could occur. We show that Pen-2 over-expression increases PS1 mRNA levels (Fig.8A) and promoter transactivation (Fig.8B) in neuronal TSM1 cells. Interestingly, Pen-2 induced increase of PS1 promoter activation was inhibited to the p53 inhibitor pifithrin-α (Fig.8B). Furthermore, we demonstrate that Pen-2 over-expression lowers PS2 promoter transactivation in a pifithrin-α sensitive manner (Fig.9). Therefore, our data suggest that PS control Pen-2 promoter transactivation by an AICD-mediated and p53-dependent mechanism (Fig.10A). In a feedback control process, Pen-2 modulates PS promoter transactivation by a p53-mediated process (Fig.10B).

Discussion

Cellular proteins homeostasis results from a complex set of regulations implying both genesis and catabolism. Most of neurodegenerative diseases are associated with increased levels of proteins that generally aggregate, giving rise to intracellular or extracellular lesions thought to be involved in the degenerative processes (Bucciantini et al., 2002). This stands in Alzheimer's disease where both extra cortical lesions called senile plaques and intracellular accumulation of abnormally phosphorylated tau are observed at a late stage of the disease (Selkoe, 1991). Senile plaques are due to the aggregation of a mix of proteins, the main component of which is Aβ. Aβ is a generic term that indeed includes a set of insoluble fragments derived from the proteolytic hydrolysis of a transmembrane protein, β-amyloid precursor protein (βAPP) by two proteolytic entities called β- and γ-secretases. γ-secretase refers to both PS-independent (Armogida et al., 2001; Wilson et al., 2002; Wilson et al., 2003; Beglopoulos et al., 2004; Lai et al., 2006) and PS-dependent activities (Herreman et al., 2000; Zhang et al., 2000). The latter was relatively recently characterized as a high molecular weight complex comprising PS1 or PS2 and nicastrin, Aph-1 and Pen-2 (Goutte, 2000; Herreman et al., 2000; Yu et al.,

2000; Zhang et al., 2000; Francis et al., 2002; Edbauer et al., 2003; Takasugi et al., 2003).

The biology of the PS-dependent γ-secretase is poorly understood although several cell biology studies emphasized the crucial role of each of the proteins in the build up of the complex. Thus, any deletion of one of the members of the complex abolishes its biological activity (Herreman et al., 2000; Zhang et al., 2000; Li et al., 2003; Ma et al., 2005). Conversely, successful reconstitution of the γ-secretase complex can only be achieved by gathering the four protein components (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). The assembly of the complex obeys a sequence of events but it is often proposed that the initial step, consists of the association of nicastrin and Aph-1 that form a sub-complex to which PS1 or PS2 incorporates (Lee et al., 2002; Hu and Fortini, 2003; La Voie et al., 2003). Then Pen-2 terminates the structure and allows PS to be processed into N- and C-terminal fragments that stochiometrically associate to yield a biologically active complex (Luo et al., 2003; Takasugi et al., 2003). This highly coordinated sequence of events implies that the levels of each of the components could be seen as rate limiting for γ-secretase formation and therefore regulate its associated phenotypes.

Several studies suggested that the catabolism of the members of the γ-secretase was drastically enhanced when proteins occur outside of the complex. Thus, reduction of Aph-1 leads to drastic reduction of PS expression (Lee et al., 2002). A few works suggested that the catabolism of the members of the complex could be regulated by proteasomal degradation (Bergman et al., 2004; Crystal et al., 2004) but a recent study indicated that these observations likely results from artifactual effect of proteasome inhibitors that non specifically up-regulate cmv-driven transcription (Dunys et al., 2006).

Relatively few data concern upstream regulation of these proteins and particularly the fact that they could be modulated at a transcriptional level. Previous studies have demonstrated the regulation of PS1 promoter transcription by Ets proteins, and particularly Elk-1 and ER81 (Pastorcic and Das, 2000; Pastorcic and

Das, 2003), c-AMP-response Element-binding protein (CREB) (Mitsuda et al., 2001) or p53 (Roperch et al., 1998; Pastorcic and Das, 2000). Concerning PS2, its transcription involves Sp1 and Egr-1 (Renbaum et al., 2003). Recently, Aph-1 and Pen-2 promoter sequences have been described (Wang et al., 2006b; Wang et al., 2006a). Aph-1 promoter appears to be regulated by HIF-1α under hypoxic conditions (Wang et al., 2006a), while Pen-2 promoter contains CREB binding domains (Wang et al., 2006b). Until now, the sequence of the nicastrin promoter is unknown and its transcriptional regulation has not been documented.

Our study clearly establishes that PS modulate Pen-2 promoter transactivation by a p53-dependent mechanism. Thus, PS depletion reduces Pen-2 expression and promoter activation. This effect is related to PS-associated activity since it is prevented by a double mutation known to abolish γ-secretase activity. This phenotype was apparently due to a γ-secretase-derived product of βAPP since depletion of βAPP mimics the one triggered by the deficiency of both PS1 and PS2 on Pen-2 expression and promoter transactivation. These data agree perfectly with our previous demonstration that AICD acted as a transcriptional regulator of p53 (Alves da Costa et al., 2006) as was demonstrated for various other proteins (Baek et al., 2002; Kim et al., 2003; von Rotz et al., 2004; Pardossi-Piquard et al., 2005; Zhang et al., 2007). This is the first demonstration that p53 could up-regulate Pen-2 transcription.

It is interesting to emphasize the fact that PS1 and PS2 trigger opposite effects on Pen-2 expression and promoter transactivation. Thus, PS1 over-expression lowers Pen-2 while PS2 exacerbates its expression and promoter transactivation. These observations fit perfectly with the opposite influence of PS1 and PS2 on p53 (Alves da Costa et al., 2006) and indirectly confirms that p53 accounts for the distinct and opposite effect of PS1- or PS2 on Pen-2. Furthermore, this scheme indicates two distinct loops of regulation of PS1 (anti-apoptotic) and PS2 (pro-apoptotic) phenotypes. Thus, previous studies demonstrated that PS1 lowers p53 (Roperch et al., 1998; Mitsuda et al., 2001). Here we show that: 1) p53 increases Pen-2 and 2) Pen-2 modulates PS1 promoter transactivation in a p53-dependent manner. Therefore, PS-1

modulation of Pen-2 ultimately leads to a p53-dependent down-regulation of its own expression (Fig.10). This feed back loop also stands for PS2 that increases p53 (Alves da Costa et al., 2002; Alves da Costa et al., 2006) and increases Pen-2 (present study) thereby controlling its expression (Fig.10). The opposite phenotype triggers by PS1 and PS2 adds support to previous studies suggesting that these proteins could indeed display their own function and elicit their specific pharmacological spectrum (Chen et al., 2003; Lai et al., 2003; Gu et al., 2004).

The present study has several conceptual implications. First, the work identifies p53 as a common effector modulating the transcriptional regulation of various members of the γ-secretase complex. Second, this is the very first indication that a member of the γ-secretase complex could participate in the transcriptional regulation of another member of this complex. Third, we demonstrate that PS1 and PS2 distinctly influence Pen-2 via an AICD- and p53-dependent mechanism, suggesting that the generic term of γ-secretase likely refers to various complexes with specific composition harboring various pharmacological functions. Finally, we show that there exist feed-back loops of regulations by which any altered phenotypes triggered by abnormal modifications of PS1 or PS2 expression could be retro-controlled and "buffered" by Pen-2. It is interesting to note that this feed back control of PS promoter transactivation by Pen-2 is mediated via p53 by a γ-secretase-independent mechanism (Dunys et al., 2007). Overall, this study shows that proteins of the complex are not only associated in post-traductional events but could also be intimately linked by upstream events implying a transcriptional control of their expression by p53 via AICD-dependent and AICD-independent mechanisms.

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Legends

Fig.1: The depletion of both PS1 and PS2 lowers Pen-2 expression and decreases the transactivation of its promoter. A) Endogenous Pen-2 and β-tubulin (loading control) immunoreactivities were analyzed in wild-type ($PS^{+/+}$) and PS-deficient fibroblasts ($PS^{-/-}$) by western blot as described in the Methods. B) Pen-2 promoter transactivation was analyzed in the indicated cell lines as described in the Methods. Bars are the means ± S.E.M of 15 independent determinations and are expressed in percent of control $PS^{+/+}$ fibroblasts. p values compares luciferase activity to that obtained in $PS^{+/+}$ cells. C) $PS^{-/-}$ fibroblasts were transfected with both PS1 and PS2 cDNA using Amaxa protocol as described in the methods. Endogenous Pen-2 expression was assessed as above with PNT-2 antibody and transfection efficiencies were established using anti-PS1-Nter and anti-PS2-Cter antibodies, respectively as described in the Methods.

Fig.2: PS1 and PS2 over-expression triggers opposite effect on Pen-2 promoter transactivation. A) Stably transfected HEK293 cells over-expressing empty cDNA (Mock), PS1 or PS2 were analyzed for their PS1 and PS2 expression as well as for endogenous Pen-2 immunoreactivity by western blot using anti-PS1-Nter, anti-PS2-Cter and PNT2 antibodies, respectively. B) The indicated cell lines were co-transfected with both Pen-2-promoter-luciferase (hpPen-2–luciferase) and a β-

galactosidase (to normalize the transfection efficiencies) reporter gene constructs then Pen-2 promoter transactivation was measured as described in the Methods. Bars are the means ± S.E.M of 8 independent determinations. p values compare luciferase activity to that obtained in mock-transfected cells.

Fig.3: Pen-2 promoter transactivation is reduced by PS1 mutations and increased by AICD. A) Stably transfected HEK293 cells over-expressing empty cDNA (Mock), wild-type PS1 (wtPS1), Asp->Ala257/Asp->Ala385-PS1 (DD-PS1) or PS2 were analyzed for their Pen-2 promoter transactivation as described in the Methods. B,C), Mock-transfected HEK293 were transiently transfected with empty pcDNA3 vector or AICD (C59) then Pen-2 expression (B) and promoter transactivation (C) were measured as described in the Methods. Bars in A and C are the means ± S.E.M of eight (A) or six (B) independent determinations and are expressed as control luciferase activity (taken as 100) obtained in Mock-transfected cells. p values compare luciferase activity to that obtained in mock-transfected cells. ns: non statistically significant.

Fig.4: The depletion of βAPP lowers Pen-2 expression and decreases the transactivation of its promoter. A,B) Endogenous Pen-2 and actin (loading control) immunoreactivities were analyzed by western blot in wild type (WT) and βAPP-deficient ($APP^{-/-}$) fibroblasts ($PS^{-/-}$) as described in the Methods. Bars in B represent densitometric analyses of endogenous Pen-2 immunoreactivity in 5 independent experiments and are expressed as percent of Pen-2 expression recovered in WT fibroblasts. C) Pen-2 promoter transactivation was analyzed in the indicated cell lines as described in the Methods. Bars are the mean ± S.E.M of three independent determinations. p values compare luciferase activity to that obtained in WT fibroblasts.

Fig.5: Effects of PS1 and PS2 on Pen-2 expression are lowered by the p53 inhibitor pifithrin-α. Stably transfected HEK293 cells over-expressing empty pcDNA3 (Mock),

wild-type PS1 (wtPS1), wild-type PS2 (wtPS2) or mutated PS1 (DD-PS1) were co-transfected with Pen-2-promoter-luciferase and β-galactosidase reporter gene constructs then treated for 16 hours with or without pifithrin-α (10μM, PFTα). Pen-2 promoter transactivation was analyzed as described in the Methods. Bars are the means ± S.E.M of four to six independent determinations. p values compare luciferase activity in a given cell type to that obtained in absence of PTFα.

Fig.6: Pen-2 expression, promoter transactivation and mRNA levels are decreased by p53 deficiency. Fibroblasts deficient for $p19^{Arf}$ ($p19^{Arf-/-}$) or for both $p19^{Arf}$ and p53 ($p19^{Arf-/-}p53^{-/-}$) were analyzed for their endogenous Pen-2 immunoreactivity (A,B). Panel B represents the densitometric analysis of Pen-2 immunoreactivity expressed as percent of control expression observed in $p19^{Arf-/-}$ fibroblasts and are the means ± S.E.M of four independent experiments. C,D) Pen-2 promoter activation (C) and Pen-2 mRNA levels (D) were monitored using the Pen-2-promoter-luciferase reporter gene construct and by real-time quantitative PCR as described in the Methods. Bars are the means ± SEM of 9 (C) and 3-4 (D) independent determinations. p values compare $p19^{Arf-/-}p53^{-/-}$ and $p19^{Arf-/-}$ fibroblasts.

Fig.7: p53 complementation increases Pen-2 expression and promoter transactivation in $p19^{Arf-/-}p53^{-/-}$. $p19^{Arf-/-}p53^{-/-}$ fibroblasts were transiently transfected with empty pcDNA3 vector (DNA3) or p53 cDNA using Amaxa protocol as described in the Methods then Pen-2 expression (A,B) or promoter transactivation (C) were analyzed as described in the Methods. Bars are the means ± S.E.M of 3-5 (B) or 4 (C) independent determinations. p values compare Pen-2 expression or luciferase activity to those observed in mock-transfected $p19^{Arf-/-}p53^{-/-}$ fibroblasts.

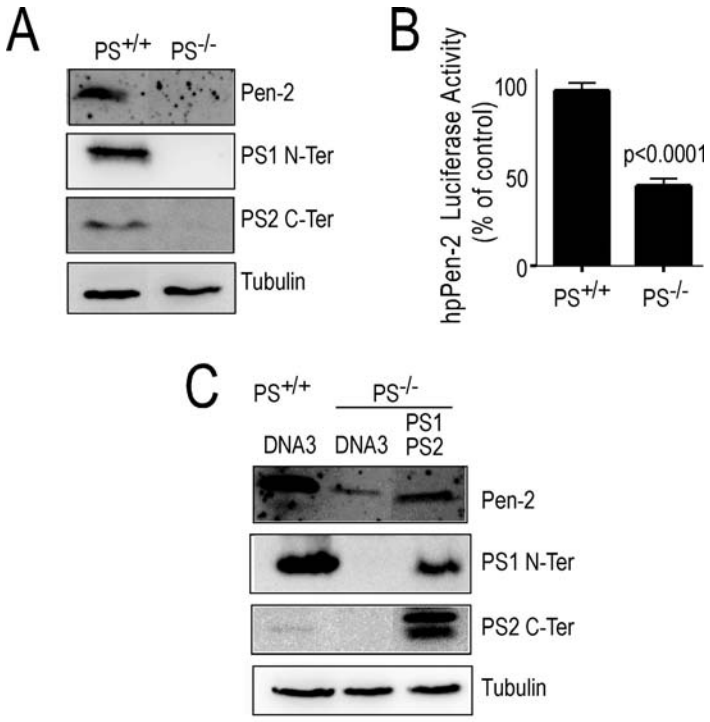
Fig.8: Pen-2 over-expression triggers pifithrin-α-sensitive increase of PS1 promoter transactivation in neurons. A) Stably transfected TSM1 neurons over-expressing Pen-2 were transfected with mouse PS1-promoter-luciferase reporter gene construct.

Twenty-four hours after transfection, cells are treated with PFTα (10μM) or with a control p53 inactive analog of PFTα (PC) then PS1 promoter transactivation was analyzed as described in the methods. B) PS1 mRNA levels were determined by real-time quantitative PCR analysis in the indicated cell line. Bars are the means of 6 (A) or 4 (B) independent determinations for promoter and real-time PCR analyses, respectively. p values compare luciferase activity or mRNA levels to those obtained in PC-treated mock-transfected cells.

Fig.9: Pen-2 over-expression lowers pifithrin-α-sensitive PS2 promoter transactivation in neurons. Stably transfected TSM1 neurons over-expressing Pen-2 were transfected with mouse PS2-promoter-luciferase reporter gene construct. Twenty-four hours after transfection, cells are treated with or without pifithrin-α (PFTα, 10μM) then PS2 promoter transactivation was analyzed as described in the methods. Bars are the means ± SEM of six independent determinations. p values compare luciferase activity to the one obtained in PC-treated mock-transfected cells.

Fig.10: Schematic representation of cross-regulation between PS, p53 and Pen-2

A) Scheme of the pathways linking PS1, PS2, AICD, p53 and Pen-2. As we previously described (Alves da Costa et al., 2006), PS1 lowers p53 while PS2 increases p53. Both proteins functionally interact but PS2 is dominant for the p53-dependent pro-apoptotic phenotype (PS2>>PS1) (Alves da Costa et al., 2006). AICD positively modulates p53 (Alves da Costa et al., 2006) and p53 increases Pen-2 transcription (present study). Conversely, there exists a feedback loop by which Pen-2 down-regulates p53 (Dunys et al., 2007). Thus, when Pen-2 expression is increased (B), p53 is lowered. Because p53 is a transcriptional repressor of PS1, Pen-2-induced lowering of p53 leads to increased levels of PS1 and concomitantly enhances the transcription of PS1 (present study).



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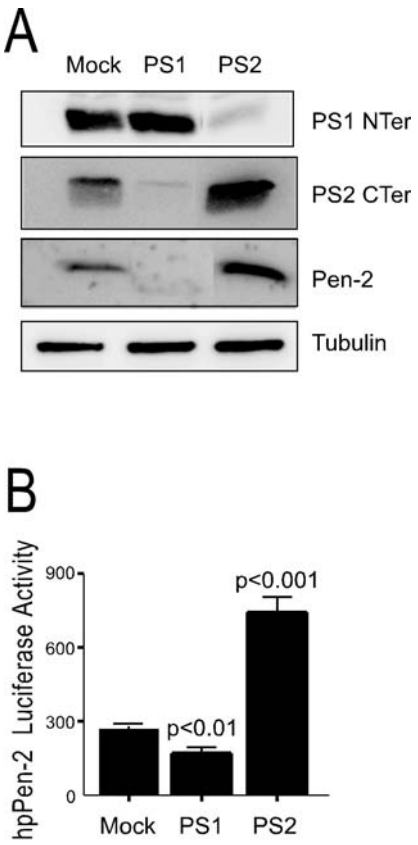

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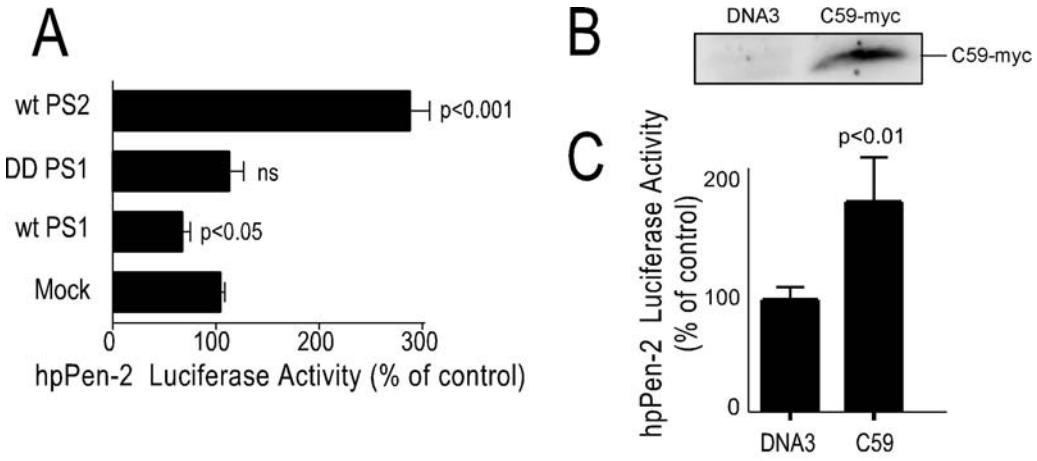

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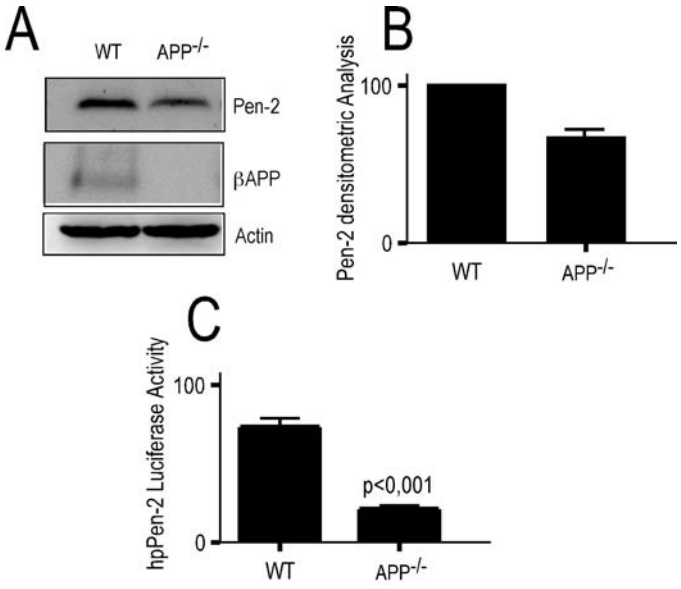

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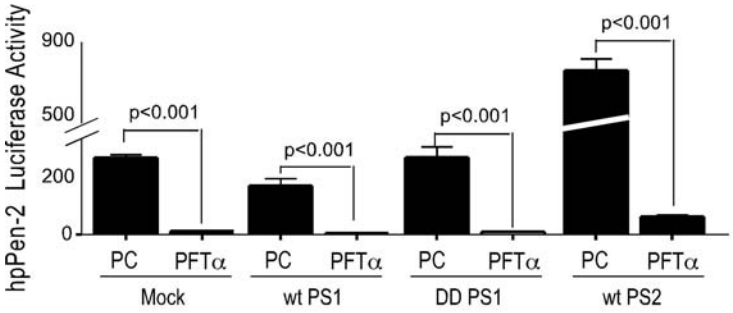

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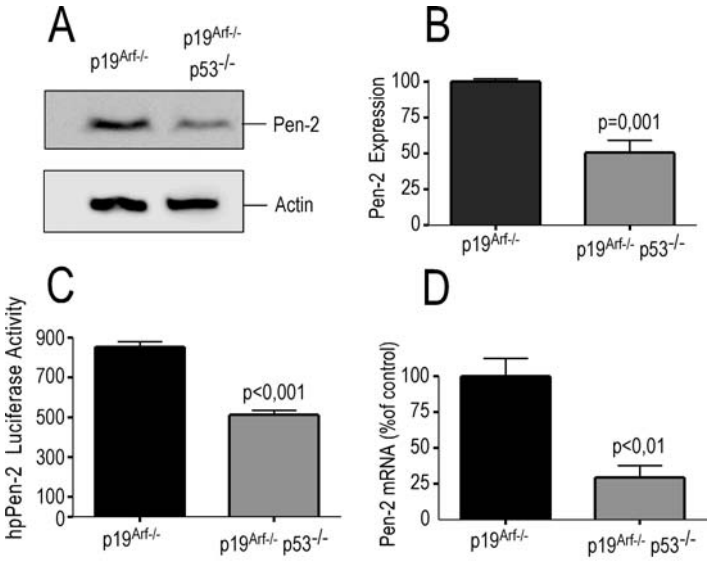

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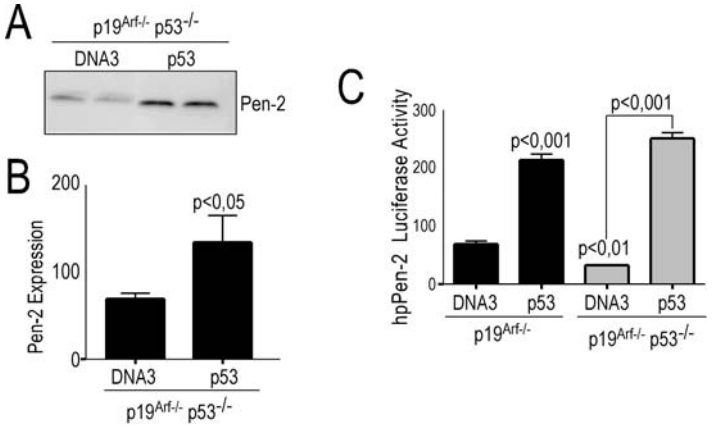

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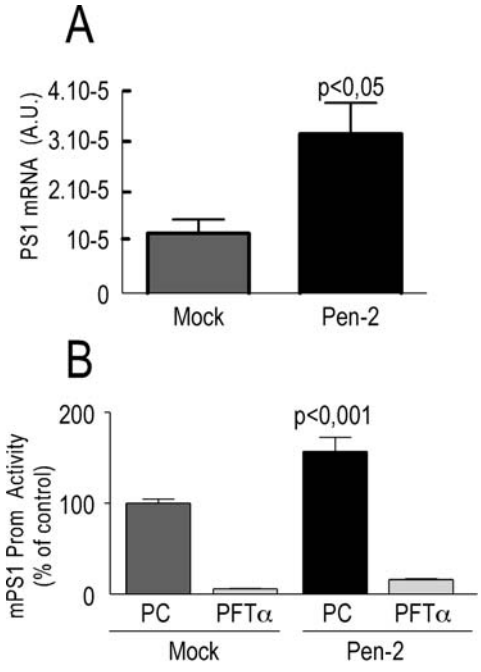

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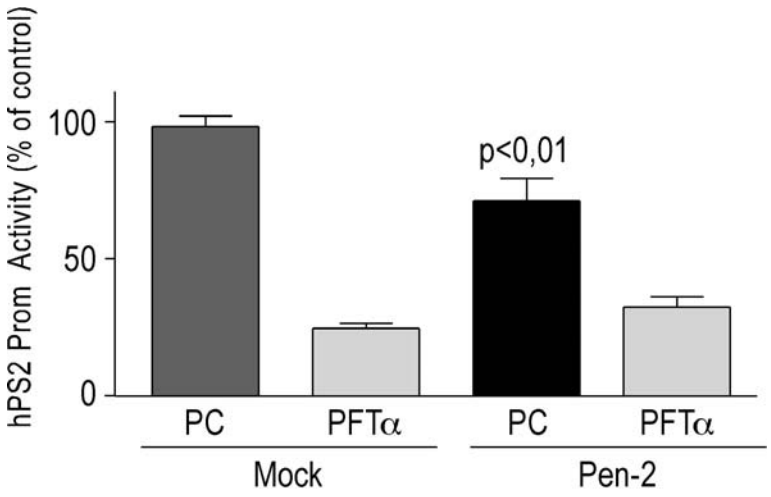

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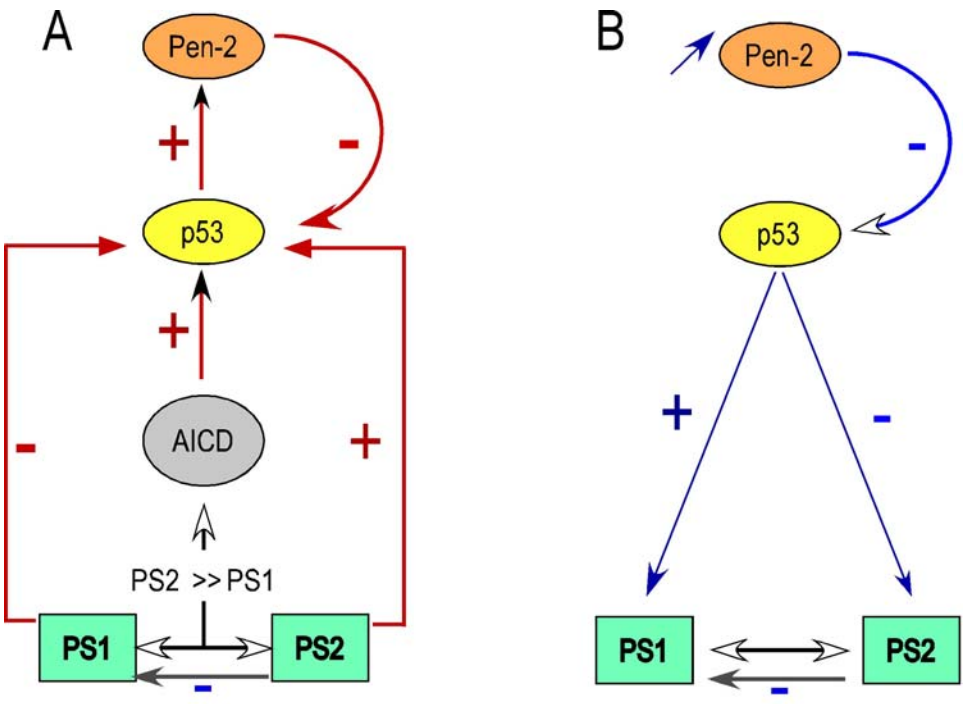

Fig.10: Dunys et al.

Article 8

Pardossi-Piquard R., Dunys J., **Giaime E.**, Guillot-Sestier M-V, St. George-Hyslop P., Alves da Costa C., and Checler F.

"p53-dependent control of cell death by nicastrin: lack of requirement for presenilin-dependent γ-secretase complex."

Soumis pour publication

p53-dependent control of cell death by nicastrin: lack of requirement for presenilin-dependent γ-secretase complex

Running title: Nicastrin-associated antiapoptotic phenotype

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Abstract

Nicastrin (NCT) is a component of the presenilin-dependent γ-secretase complex that liberates Aβ peptides from the β-Amyloid Precursor Protein. Several lines of evidence indicate that the members of this complex could also contribute to the control of cell death. Here we show that over-expression of NCT increases the viability of human embryonic kidney (HEK293) cells and decreases staurosporine (STS)- and thapsigargin (TPS)-induced caspase-3 activation in various cell lines from human and neuronal origins by Akt-dependent pathway. NCT lowers p53 expression, transcriptional activity and promoter transactivation and reduces p53 phosphorylation. NCT-associated protection against STS-stimulated cell death was completely abolished by p53 deficiency. Conversely, the depletion of NCT drastically enhances STS-induced caspase-3 activation and p53 pathway and favored p53 nuclear translocation. We examined whether NCT protective function depends on presenilin-dependent γ-secretase actiivty. First, a 29-amino acid deletion known to reduce NCT-dependent Aβ production did not affect NCT-associated protective phenotype. Second, NCT still reduces STS-induced caspase-3 activation in fibroblasts lacking presenilin 1 and presenilin 2. Third, the γ-secretase inhibitor DFK167 did not affect NCT-mediated reduction of p53 activity. Altogether, our study indicates that NCT controls cell death via PI3-kinase/Akt and p53-dependent pathways and that this function remains independent of the activity and molecular integrity of the γ-secretase complex.

Key Words: Nicastrin, Presenilins, Caspase-3, p53, Akt, NFκB, γ-secretase complex, knockout fibroblasts.

Abbreviations: NCT, Nicastrin; TSM, Telencephalon Specific Murine; PS, Presenilin; Aph-1, Anterior pharynx defective-1; Pen-2, Presenilin enhancer-2; MEF, Mouse Embryonic Fibroblasts; STS, Staurosporine; Ac-DEVD-al, acetyl-Asp-Glu-Val-Asp-aldehyde; TUNEL, Terminal dUTP Nick-end Labeling; PARP, Poly-(ADP)-Ribose-Polymerase; Mdm2, Mouse double minute 2, siRNA, small interference RNA; DFK, DiFluoro-methyl Ketone; TPS, thapsigargin.

Introduction

Alzheimer's disease (AD) is the most common age-dependent cause of dementia. This disease is mainly of sporadic origin but a few monogenic familial forms characterized by an early onset have been identified. These genetic forms are associated with mutations on genes coding for the amyloid precursor protein (βAPP), presenilin 1 (PS1) and 2 (PS2) (1). All Familial Alzheimer's Disease (FAD) cases are associated with a modulation of Aβ production with selective increase in the production of amyloid β-peptide (Aβ) or N-terminally truncated Aβ-species ending at residue 42.

Aβ peptides derive from their precursor (β-amyloid precursor protein) through proteolytic processing by β- and γ-secretases that are responsible for the liberation of the N- and C-termini, respectively (2). β -secretase activity is harbored by a membrane-bound aspartyl protease referred to as BACE1 (β-site APP cleaving enzyme), memapsin 2 or ASP2 (3). The γ-secretase activity could be presenilin (PS)-dependent (4) or PS-independent (5, 6). The PS-dependent activity has been described as a high molecular weight complex composed of at least four distinct proteins, namely PS1 or PS2, nicastrin (NCT), Aph-1 and Pen-2 (7).

The amyloid hypothesis proposes that the overproduction of Aβ peptide at least contributes to a cascade of events (8) that culminates, at late stages of the disease, in neuronal dysfunction and cell death. A growing number of anatomical evidences suggest that exacerbated cell death characterizes AD-affected brains (9-11). Thus, several groups have demonstrated increased DNA fragmentation and caspase activation in tissue sections of brains of Alzheimer's disease patients (12, 13). Consistent with these histochemical observations, it is noteworthy that βAPP, PS1 and PS2 behave as substrates of the pro-apoptotic effector enzyme, caspase-3. (14-16). Furthermore, FAD mutations not only affect Aβ42 production but also increase cell death vulnerability (17). Whether the modulation of cellular Aβ levels and cell vulnerability are directly linked remains questioned. However, numerous reports have shown that Aβ *per se* could trigger cell toxicity and apoptosis, the extent of which appeared exacerbated by the two amino-acids C-terminal extension harbored by Aβ42 (18).

Cell biology approaches allowed to establish that wild type PS1 and PS2 differently modulate cell death in response to apoptotic stimuli. Thus, lowering PS1 expression by antisense cDNA triggers increased apoptosis (19), suggesting a protective role for PS1. Conversely, PS2 was reported to be pro-apoptotic in several cell types (for review see (17)). Interestingly, the tumor suppressor p53 behaves as a common denominator of both PS1- and PS2-associated functions. Thus, p53 down-regulates PS1 expression, likely at the transcriptional level (20) while PS1 lowers p53 expression, activity and mRNA levels (21). Furthermore, we have demonstrated that PS2 elicits a p53-dependent pro-apoptotic response that is exacerbated by FAD mutations (22). Again, a link between Aβ production and the

control of p53-dependent cell death has been suggested by a study showing that Aβ42 could increase cell death in a p53-dependent manner (23).

Unlike PS, very little is known about the contribution of the other protein components of the PS-dependent γ-secretase complex. Recently, Xia and co-workers documented an increased cell death in zebrafish devoid of Pen-2 (24). There again, the p53 pathway appears involved in the cell death pathway controlled by Pen-2 (24). We recently showed that both Aph-1 and Pen-2 controlled cell death in mammalian cells by modulating the p53-dependent pathway (25).

Here we report for the first time that the over-expression of wild type NCT lowers STS and TPS-induced caspase-3 activation and decreases p53 expression and phosphorylation, p53 transcriptional activity and p53 promoter transactivation. Conversely, the depletion of NCT in mouse embryonic fibroblasts (MEF) or by siRNA strategy in SH-SY5Y cells triggers the opposite phenotype. We also show that NCT-mediated reduction of STS-induced caspase-3 activation and cell death enhancement involves the Akt pathway but not the NFκB signaling and were fully abolished by p53 deficiency. Finally, of most interest, we establish that NCT displays its protective phenotype in $PS^{-/-}$ fibroblasts and that DFK167 did not interfere with NCT-associated protective function, indicating that the p53-dependent NCT anti-apoptotic phenotype does not require PS-dependent γ-secretase complex molecular integrity and activity.

Materials and Methods

Cell culture and transfections

Human Embryonic Kidney 293 cells (HEK293) expressing wild-type nicastrin (NCT-WT) or its mutants D336A/Y337A-NCT (NCT-AA), Δ312-340-NCT (NCT-Δ) (26) were obtained and cultured as previously reported (27). Telencephalon Specific Mouse 1 neurons and SH-SY5Y neuroblastoma cells (TSM1) were cultured as previously detailed (22,28) were cultured as previously described. Mouse Embryonic Fibroblasts (MEF) devoid of PS1 and PS2, $p19^{arf}$ and $p19^{arf}$ p53 and NCT were obtained (29-32) and cultured (21, 33) as previously reported. Transient transfections were carried out by means of DAC30 (Eurogentec) or Lipofectamine 2000 (Invitrogen) according to manufacturer's conditions as previously detailed (22). In some cases, fibroblasts were transfected by means of the mouse embryonic fibroblasts NucleofectorTM kit (Amaxa Biosystems, Koeln, Germany) as described (34).

Flow Cytometry analysis.

HEK293 cells were grown in 6-well plates and treated for 16 h at 37°C in the presence or in the absence of STS (2μM, STS, Sigma). Cells were harvested, pelleted by centrifugation at 1,000 x g for 10 min at 4°C, gently resuspended in 500μl of 0,1% natrium citrate buffer containing 50μg/ml of propidium iodide (PI), and incubated overnight under agitation. The

PI fluorescence of individual nuclei was measured by using a FACScan flow cytometer (program CELLQUEST, Becton Dickinson). Red fluorescence due to PI staining of DNA was expressed on a logarithmic scale simultaneously to the forward scatter of the particles. Fifty thousand events were counted on the scatter gate. All measurements were performed under identical conditions. This technique allows the discrimination of populations of apoptotic nuclei from debris and non-viable cells and also from diploid nuclei that show higher fluorescence staining. The number of apoptotic nuclei is expressed as a percentage of the total number of events.

siRNA inactivation of endogenous NCT

siRNA duplexes targeting NCT (provided by Dr. Gang Yu) were selected for their capability to inactivate NCT gene. SH-SY5Y neuroblastoma cells were cultured until they reached 90% of confluency, then transfected with the siRNA duplex for 16 hours using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were then split as 1:3 and were subjected to a second round of transfection. Twenty-four hours later, cells were treated for 2 hours with or without STS (1μM), then scrapped and analyzed for their caspase-3 activity as described below. Protein silencing was monitored by western blot as described below.

Cell treatments for caspase activity measurements

MEF cells, SH-SY5Y neuroblastoma cells, HEK293 cells and TSM1 neurons were cultured in 6-well plates and then treated with STS (1µM or 2µM) or thapsigargin (1µM). In some experiments, HEK293 cells were pre-incubated for 24h at 37°C with Ac-DEVD-al (caspase inhibitor, 100µM, Sigma) before stimulation of apoptosis by STS. When indicated, MEF cells were pre-treated with LY294002 (10µM, 30 min) (VWR, Leuven, Belgium) then treated with STS (1µM) for 4 hours. Cells were harvested, pelleted by centrifugation at 4,000 X g for 5min then assayed for their caspase-3-like activity as extensively detailed (22).

Tunel analysis of cell death.

$p19^{arf\ -/-}$ and $p19^{arf\ -/-}$ $p53^{\ -/-}$ MEF cells were transfected with empty $pcDNA_3$ vector or NCT cDNA by means of the mouse embryonic fibroblasts Nucleofector™ kit according to the manufacturer's instruction (Amaxa Biosystems, Koeln, Germany) and cultured in polylysinated six-wells plates. Twenty-four hours after transfection, cells were treated or not with staurosporine (2µM, 4h) then fixed for 30 min with 4% paraformaldehyde, rinsed in PBS, permeabilized overnight with 70% ethanol, and then processed for the dUTP nick-end labeling (Tunel) technique as described previously (35). Staining was assessed with peroxidase-conjugated antibody and revealed with a diaminobenzidine substrate as described (35). Fragmented DNA labeling corresponds to black spots. The visualization of the totality of the cells was carried out with erythrosin B.

p53 transcriptional activity and p53 promoter transactivation.

p53 transcriptional activity was measured by means of pG13-luciferase (PG13) construct (kindly provided by Dr. B. Vogelstein) which harbors the genomic DNA consensus sequence targeted by p53 (36). p53 promoter transactivation was measured with a construct (kindly provided by Dr. M. Oren) bearing the murine p53 promoter sequence in frame with luciferase (37). HEK293 cells were co-transfected with 1µg of pG13-luciferase or promoter p53-luciferase cDNA and 1 µg of a β-galactosidase transfection vector to normalize transfections efficiencies. In some experiments, cells were treated for 16 hours with DFK167 (50µM). Forty-eight hours after transfection, luciferase and β-galactosidase activities were measured as previously described (22).

Analysis of nuclear translocation of p53

$NCT^{+/+}$ or $NCT^{-/-}$ fibroblasts were submitted to a cellular fractionation process yielding nuclear and cytosolic fractions (22). For each fraction, 25 μg of proteins were separated on 12% Tris-glycine gels, wet-transferred on nitrocellulose and probed with an anti-p53 antibody as described below.

Co-immunoprecipitation experiments

Wild type NCT-over-expressing HEK293 human cells were gently homogenized in lysis buffer (10mM Tris, pH 7.5, 150mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5mM EDTA) and centrifuged at 14,000 x rpm in order to remove cellular debris. 500µg of proteins were incubated overnight with PBS, 20μl of protein A sepharose and 2μg of anti-V5- or anti-p53 mouse antibodies. After centrifugation (14,000 x rpm), pellets were washed 3 times with RIPA buffer (10mM Tris, 50mM EDTA, 1,5M NaCl). Samples were resolved on 8% SDS-PAGE gels. NCT and p53 were detected respectively with anti-V5 monoclonal and anti-p53 polyclonal antibodies as described bellow.

Analysis of protein expressions

Equal amounts of protein (50µg) were separated on 8% (detection of NCT, poly-ADP-ribose polymerase (PARP and Mdm2), 10% (p53 and β-tubulin) or 12% (active caspase-3, Pp53, pAkt, PS1 and PS2) SDS/PAGE gels. Proteins were then transferred to Hybond-C membranes (Amersham Pharmacia Life Science), blocked with non-fat milk or bovine serum albumin (1% TBS) and incubated overnight with the following primary antibodies: anti caspase-3 active (rabbit polyclonal, R&D System), anti-PARP (rabbit polyclonal, BD Pharmingen), anti-p53 (mouse monoclonal, Santa Cruz Biotechnology), anti-p53 CM1 (rabbit polyclonal, gift of JC Bourdon), anti-p53 phospho-Ser6 (rabbit polyclonal, Anaspec Inc, San Jose, CA), anti-Akt phosphoSer 473 (rabbit polyclonal, Cell Signaling), anti-β-tubulin (mouse monoclonal, Sigma), anti-V5 that recognizes V5-tagged-NCT (mouse monoclonal, Invitrogen), anti-NCT (rabbit polyclonal, provided by Dr Paul Fraser), anti-Mdm2 (mouse

monoclonal, provided by Robin Fahraeus), anti-PS1 and anti-PS2 were provided by Dr. W. Araki. Immunological complexes were revealed with anti-rabbit or anti-mouse peroxidase-coupled antibodies (Jackson Immunoresearch), followed by electrochemiluminescence detection (Roche). All protein concentrations were determined by the BioRad procedure.

Results

NCT over-expression increases cellular viability and lowers STS- and TPS-induced caspase-3 activation in various human and neuronal cell lines.

We have previously set up and characterized stably transfected HEK293 cells over-expressing V5-tagged wild-type NCT (NCT-WT, Fig.1A and see (27)). STS treatment of mock-transfected HEK293 cells drastically increases the number of apoptotic nuclei as measured by PI incorporation and FACS analysis (Fig.1B). This effect was drastically reduced in NCT-WT cells (Fig.1B, 55,5% of reduction, $p<0,005$, $n=6$). This result was confirmed by TUNEL analysis (double staining for terminal deoxynucleotide transferase nick end labeling), where a 58% decrease in the number of TUNEL-positive cells was observed in STS-treated NCT-WT cells when compared with STS-treated mock-transfected cells (data not shown).

Is the increased viability triggered by NCT due to a reduction of the caspase-3-dependent pathway? This question was addressed by direct measurements of caspase-3 activity and indirectly, by assessing the cleavage of PARP, a typical caspase-3 substrate. Caspase-3 activity measured by means of a fluorimetric substrate was increased by STS (Fig.1C,D) and TPS (Fig.1E) in mock-transfected HEK293 cells but to a much lesser extent in NCT-WT cells (Fig.1C-E). It is noteworthy that in all cases, this activity was fully inhibited by the caspase-3 inhibitor Ac-DEVD-al (Fig.1D). It is well documented that poly-ADP-ribose polymerase occurs as an inactive precursor (120kDa) that is proteolytically activated by caspase-3 cleavage (89kDa). Thus the ratio between PARP precursor and its active product (prec/prod) is generally a good index of caspase-3 activity. As expected from caspase-3 activation, STS treatment of mock-transfected HEK293 cells lowers PARP precursor expression and concomitantly increases PARP product (Fig.1F). By contrast, the over-expression of NCT lowers PARP product in both basal and STS-stimulated conditions, resulting in a significantly higher PARP prec/prod ratio (Fig.1G).

The protective effect of NCT is not cell specific. Thus, in TSM1 neuronal cells, the STS treatment increases both active caspase-3 immunoreactivity (Fig.2A) and activity (Fig.2B), a phenotype fully prevented by NCT over-expression (Fig.2A,B), indicating that NCT also triggers its protective function in neurons.

NCT over-expression lowers p53 expression, activity and promoter transactivation.

We recently demonstrated that PS1 and PS2 could differently control p53-dependent cell death pathway (21, 22, 38). We therefore examined whether NCT, which is part of the PS-dependent γ-secretase complex, could display its protective function through the control of the p53 pathway. First, in basal conditions, NCT-WT cells display lower p53 expression when compared to mock-transfected cells (Fig.3A). As previously described (21), STS increases p53 expression in mock-transfected cells (Fig.3A) a phenotype that is prevented by NCT expression (Fig.3A,B). NCT expression also significantly decreases p53 activity (Fig.3C) and p53 promoter transactivation (Fig.3D). Two sets of experiments suggest that NCT controls p53 activity at a post-transcriptional level. First, NCT over-expression drastically reduces p53 phosphorylation (Fig.3E). Second, the expression of Mdm2, a protein that ubiquitinates p53, thereby leading to enhancement of its degradation by the proteasome is drastically enhanced in NCT-WT cells. Clearly, NCT increases Mdm2 immunoreactivity in both basal and STS-treated conditions (Fig.3A). However, NCT did not physically interact with p53 (Fig.3F), ruling out any putative direct regulation such as the possibility that NCT might compete with Mdm2 for regulating p53.

Depletion of endogenous NCT increases p53 expression, activity and promoter transactivation.

We examined whether endogenous NCT triggers the same protective response than the one observed when NCT was over-expressed. Indeed, mouse embryonic fibroblasts (MEF) devoid of NCT display increased caspase-3 activation in response to STS (Fig.4A). This increased susceptibility to STS was accompanied by increased PARP cleavage (Fig.4B), in agreement with an enhancement of caspase-3 activity. NCT depletion enhances p53 expression and nuclear translocation (Fig.4C-E) and favors p53 activity (Fig.4F), indicating that endogenous NCT also controls the p53-dependent pathway.

In order to rule out the possibility that the pro-apoptotic phenotype observed in absence of NCT could be only due to fibroblastic clonal variations unrelated to NCT genotype, we targeted NCT expression by a siRNA strategy in SH-SY5Y. siRNA reduced NCT expression by about 70% (Fig.4G,H) and this was accompanied by an increase of caspase-3 activity (Fig.4I), confirming the NCT-associated inhibitory control of caspase-3 activation observed with NCT-deficient fibroblasts.

NCT protective function is fully dependent of p53.

Whether NCT-associated response was totally linked to p53 or could have a p53-independent component still remained to be established. Two recently designed cell lines, i.e $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts, allow examining the influence of p53 depletion on cell death process without interfering with the p53 function in cell cycle control (39). We have previously established that $p19^{arf-/-}p53^{-/-}$ fibroblasts still display caspase-3 activation in

response to STS (21). Fig.5A shows that STS also increased cell death as measured by Tunel analysis in both $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts although to a lesser extent in the latter cell system. Interestingly, NCT expression lowers STS-induced cell death in $p19^{arf-/-}$ (Fig.5B,C) but this effect was fully abolished by p53 depletion (Fig.5C). Accordingly, NCT expression lowered STS-induced caspase-3 activation in $p19^{arf-/-}$ but not in $p19^{arf-/-}p53^{-/-}$ (Fig.5D).

NCT protective function is abolished by inhibitors of the PI3K-Akt survival pathway.

One of the major molecular pathways involved in cell survival implies the activation of Akt/PKB after its phosphorylation by the PI3 kinase. Thus, several studies indicated that the PI3 kinase inhibitor LY294002 reduces Akt-mediated resistance to apoptosis. We therefore examined whether LY294002 could interfere with NCT-induced protective phenotype in fibroblasts. Interestingly, LY294002 increases caspase-3 activation in basal (compare LY and CT) and STS-stimulated (compare STS and STS-LY) conditions in both Mock- and NCT-transfected cells (Fig. 6A,B). We confirm the NCT-associated reduction of STS-stimulated caspase-3 activation (compare Mock-STS with NCT-STS in Fig.6B). However, LY294002 fully reversed the NCT-associated protective phenotype (compare LY-STS-Mock and LY-STS-NCT, not significant). This data indicates that NCT protective phenotype involves the Akt survival pathway. In order to further support this hypothesis, we directly examined the activation of Akt by determining the levels of phosphorylated Akt. As expected, STS reduced Akt phosphorylation (Fig.6C) while NCT expression increased the phosphorylated Akt expression in STS conditions (Fig.6C).

It is interesting to note that NCT lowers the promoter transactivation of p53 suggesting a transcriptional effect of NCT (see Fig.3). However, the full blockade of the NCT-mediated effect on caspase-3 by LY294002 suggested that the whole NCT-related phenotype was rather linked to a modulation of post-transcriptional events implying Akt. In order to resolve this apparent paradoxical data, we examined whether the effect of NCT on p53 promoter transactivation in fibroblasts could be due to a self-control of p53 on its own promoter activity. This possibility was assessed by the co-transfection of p53 cDNA and its promoter construct Pp53 (see methods) in p53-deficient cells. This system allows to get rid of endogenous p53 promoter and therefore, any modulation of p53 promoter transactivation could be only explained by the ability of exogenous p53 to transactivate its own promoter. Indeed, Fig.7 demonstrates that p53 expression triggers transactivation of p53 promoter in both $p19^{arf-/-}$ and $p19^{arf-/-}p53^{-/-}$ fibroblasts.

Does NCT antiapoptotic function require full molecular integrity of the PS-dependent γ-secretase complex?

NCT is part of the PS-dependent γ-secretase complex (7, 26). Whether NCT functions are fully linked to its participation to this complex or alternatively, whether NCT could trigger PS-independent phenotypes remained questionable. We took advantage of

previously described artificial mutants of NCT to examine whether the physical interaction between PS and NCT could impair NCT-mediated protective function. Thus, Yu and colleagues reported on two types of artificial NCT constructions triggering various effects on Aβ peptide production. A punctual double mutation D336A/Y337A (NCT-AA) led to a NCT variant able to co-precipitate with PS1 while conversely, a deletion mutant (Δ312-340-NCT) significantly reduced the NCT-PS1 interaction (26). Stably transfected HEK293 cells expressing both mutated NCT reduced STS-induced caspase-3 activation to similar extents than NCT-WT cells (Fig.8A). This indirectly suggested that NCT could trigger its protective function even when NCT-PS1 interaction was altered. In order to further strengthen this conclusion, we examined the ability of NCT to be protective in fibroblasts devoid of PS. Indeed, NCT displays similar reduction of STS-induced caspase-3 activation in wild-type and PS1/PS2-deficient fibroblasts (Fig.8 B-D). Finally, DFK167, a γ-secretase inhibitor that physically interacts with presenilins, did not affect NCT-induced decrease of p53 activity in stably transfected HEK293 cells (Fig.8E). This clearly confirms that NCT-induced anti-apoptotic phenotype is independent of PS and does not require the structural integrity of the PS-dependent γ-secretase complex.

Discussion

Apoptosis or programmed cell death is a normal and important process to control cell population during development. Failure in apoptosis could lead to abnormal proliferation and cancer, while excessive apoptosis is observed in most of neurodegenerative diseases. Several lines of evidence indicated that sporadic cases of AD are associated with enhanced apoptosis (9-11) and that this pro-apoptotic phenotype could be exacerbated in familial cases of AD by mutations harbored by βAPP or PS1/2. Thus, βAPP and PS behave as cellular targets of caspases (14-16), the pro-apoptotic effectors which expression is increased in post-mortem Alzheimer's disease brain tissues (12, 13). This supports the hypothesis that caspase-mediated apoptotic mechanisms may contribute to the neuronal loss observed in Alzheimer's disease brain (12, 41).

Interestingly, we show here that cells over-expressing NCT, a major partner of PS in the PS-dependent γ-secretase complex (26), displays an anti-apoptotic phenotype. First, the over-expression of wild-type NCT increases cell viability in STS-stimulated conditions as shown by FACS and *in situ* tunnel analyses. Second, the over-expression of wild-type NCT lowers STS- and TPS-induced caspase-3 activation and expression in HEK293 cells, TSM1 neurons and fibroblasts.

Is the over-expression approach responsible for a physiologically irrelevant accumulation of unfolded NCT that would account for the observed alteration of cell death? This question is worth raising since NCT is mainly expressed in the endoplasmic reticulum and it is reasonable to envision that a putative overload of the protein could have triggered endoplasmic reticulum stress. It is however very unlikely for both conceptual and

experimental reasons. First, on a theoretical point of view, the adaptative UPR response (unfolded protein response) is activated to up-regulate ER-resident chaperons and to augment ER-folding capacity. If these adaptative mechanisms are not sufficient to reduce unfolded proteins, as is the case when proteins are overloaded, an apoptotic response is initiated (42). In most cases, this leads to a pro-apoptotic phenotype. This does not fit with our study describing a protective phenotype triggered by over-expressed NCT. Second, in a set of experiments aimed at determining the endogenous contribution of NCT in the control of cell death, we established that fibroblasts devoid of NCT displayed enhanced susceptibility to STS as indicated by a potentiation of caspase-3 activation and PARP cleavage. This was not due to clonal variation unrelated to NCT genotype since we established that SH-SY5Y in which NCT expression had been reduced by siRNA targeting also exhibited increased susceptibility to STS. Overall, these data strongly suggest that endogenous NCT contributes to the control of cell death by down-regulating caspase-3 activity in fibroblasts, human cells and in neurons. It should be noted that these data agree well with the demonstration that mouse embryos lacking NCT exhibit specific apoptotic stigmata in heart and brain (43).

A question remained concerning the mechanism by which NCT could control cell death. Three lines of data firmly suggest that the NCT-associated protective function occurred through the modulation of the p53-dependent pathway. First, the over-expression of NCT induced a significant decrease in p53 promoter transactivation, protein expression and phosphorylation and transcriptional activity in HEK293 cells. Second, NCT-deficient fibroblasts display significant increases in p53 expression and transcriptional activity, suggesting that endogenous NCT also controls p53. Third, the depletion of p53 fully prevented the NCT-associated protection against STS-induced cell death and caspase-3 activation. The above data would suggest that NCT exerts both transcriptional and post-transcriptional control of p53. However, a series of experiments indicated that NCT only affects p53 at a post-transcriptional level. First, NCT increased the expression of Mdm2, a protein involved in p53 ubiquitination and proteasomal degradation (44). Second, NCT expression was associated with a down-regulated expression of phosphorylated p53. Third, NCT reduced p53 nuclear expression. The above phenotypes were not due to a physical interaction between NCT and p53 that do not co-immunoprecipitate.

We attempted to delineate the cellular pathways by which NCT could control p53 by examining the putative involvement of upstream molecular effectors known to functionally interact with this tumor suppressor. It has been consistently proposed that the p53 pathway is under the control of the PI3K/Akt survival pathway (45). Thus, Akt has been shown to regulate p53 pathway at a post-transcriptional level by influencing its Mdm2-dependent stability (46), thereby leading to p53 inhibition. In order to delineate whether the NCT-associated phenotype involved the PI3-Kinase/Akt survival pathway, we examined the effect of the PI3-kinase inhibitor LY294002 on the NCT-associated phenotype. LY294002 fully

abolished the NCT-induced reduction of STS-stimulated caspase-3 activation. Interestingly, NCT-associated increase of Akt phosphorylation confirmed the direct activation of Akt by NCT.

Strikingly, our study evidenced an apparent discrepancy between the ability of NCT to down-regulate p53 promoter transactivation while the NCT-associated phenotype appeared totally blocked by LY294002, suggesting a modulation at a post-transcriptional level. This prompted us to examine whether p53 itself could be able to up-regulate its own transcription in fibroblasts. Indeed, we showed that it was the case since co-expression of p53 and its promoter construct in p53-deficient cells increases the p53 promoter transactivation. This agrees well with a previous study identifying a p53-responsive element in the p53 promoter and showing that p53 regulated its own transcription in NIH3T3 and DP15 cells (49). Overall, our study establishes for the first time that NCT-mediated post-transcriptional modulation of p53 involves the Akt pathway and that the NCT-associated modulation of p53 promoter transactivation was likely due to the p53 control of its own promoter.

It has been demonstrated that NCT, together with Aph1, Pen2, and presenilin 1 (PS1) or 2 (PS2), contributes to the formation of a high molecular weight enzymatic complex involved in the intramembranous proteolysis of several transmembrane proteins including βAPP (for review see (7)). Several of these proteins were previously shown to modulate the p53-dependent pathway. Thus, PS1 and PS2 cross-talk to modulate p53 at a transcriptional level, via the production of the γ-secretase-derived C-terminal fragment of βAPP referred to as AICD (21, 22). Furthermore, in agreement with recent studies carried out *in vivo* in mice and in the zebrafish (24, 50), we recently documented an anti-apoptotic phenotype associated with Aph-1 and Pen-2 and we showed that it was linked to the ability of both proteins to lower the p53-dependent pathway (16). Interestingly, in this case, the protective function elicited by Aph-1 and Pen-2 was dependent of the molecular integrity of the γ-secretase complex but fully independent of its catalytic activity (16). These data suggested that members of the PS-dependent γ-secretase complex, besides their canonical function in building up the catalytic activity, could also harbor functions unrelated to its enzymatic property.

The present study fully supports the idea that some of these proteins could have a functional role outside the PS-dependent γ-secretase complex. Three distinct lines of data support this conclusion. First, we examined the potential of mutated NCT variants to protect cells from apoptotic stimulus. A missense mutation (NCT-AA) was previously shown to increase Aβ40 and Aβ42 productions while a deletion mutant (Δ340-NCT) had an impaired ability to physically interact with PS, thereby leading to reduced production of Aβ (26). In both cases, one would have expected these NCT mutation/deletion to alter the NCT-associated antiapoptotic phenotype if the latter was linked to the presence of NCT into the complex. Thus, the NCT-AA-mutation should have enhanced the anti-apoptotic phenotype if linked to γ-secretase activity while conversely; Δ340-NCT should have impaired NCT-

mediated function. However, none of these mutations affected the NCT-mediated protective function. Second, DFK167, a PS-directed γ-secretase inhibitor did not modify NCT-induced protective response. Third, NCT anti-apoptotic function appeared independent of the presence of PS since NCT-anti-apoptotic phenotype was similar in PS-containing and PS-deficient fibroblasts. Overall, our data clearly show that NCT-associated antiapoptotic phenotype was independent of the PS-dependent γ-secretase complex and therefore, of its catalytic activity.

In conclusion, our study demonstrates for the first time at a cellular level, an implication of NCT in the control of cell death and an NCT-mediated and Akt-dependent modulation of the p53 pathway, at a post-transcriptional level. The demonstration that this function remains independent of the γ-secretase activity underlines the fact that very much remains to be explored to understand the likely wider than anticipated physiological spectrum of each of the members of the γ-secretase complex, inside and outside this complex. This obviously adds an increment in the complexity of studying these protein effectors at an integrated cellular level and to delineate their function, *in vivo*.

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Legends to figures

Fig.1: Anti-apoptotic phenotype of wild-type NCT-expressing HEK293 cells. A, Western blot analysis of V5-tagged nicastrin immunoreactivity in Mock-transfected HEK293 cells (Mock) and in cells stably over-expressing wild-type V5-tagged NCT (NCT-WT). B, Representative illustration of PI incorporation measured in the indicated cell lines in control (CT) and STS-stimulated (STS, 2μM, 16h) conditions by FACS analysis (similar data were obtained in three independent experiments). The number of apoptotic nuclei is expressed as the percentage of the total number of events as detailed in Methods. C-E, Caspase-3 activity was fluorimetrically recorded in Mock-transfected (Mock) and in NCT-WT cell lines, in presence of STS (STS, 2μM, C,D) or thapsigargin (TPS, 1μM, E), for the indicated time incubations (C) or 16 hours (D,E). In D, cells were preincubated for 24 hours where indicated with Ac-DEVD-al (DEVD, 100μM). Values in C are the means ± SEM of 4-10 independent determinations. Bars in D and E are the means ± SE of 10-20 or 3 independent determinations, respectively. F, Western blot analysis of PARP immunoreactivity in Mock- and NCT-WT cells, in control (CT) and STS-stimulated (STS, 2μM, 16h) conditions. Note that PARP precursor (band at 120-kDa, prec) is converted in STS-stimulated condition into a PARP product (band at 89-kDa, prod). In A and F, protein charge was controlled by β-tubulin analysis. In G, histograms represent the densitometric analysis of PARP-like immunoreactivities in STS-stimulated conditions. The ratio precursor/product (prec/prod) is expressed as the percent of control STS-treated mock-transfected cells. Bars are the means ± SE of 8 independent determinations. *p<0,05; **p<0,01; ***p<0,001.

Fig.2: Protective effect of NCT in TSM1 neurons. Western blot analysis of V5-tagged-NCT and active caspase-3 expressions (A) and caspase-3 activity (B) in TSM1 neurons transiently transfected with empty vector (pcDNA3) or wild-type NCT-cDNA (NCT-WT) in control (CT) or STS-(STS, 1μM, 2h) conditions. Bars in B are the means ± SE of 6 independent determinations, respectively. *p<0,05.

Fig.3: NCT decreases expression, activity and promoter transactivation of p53. A, Western blot analysis of p53 and Mdm2 expressions, in control (CT) and STS-stimulated (STS, 2μM,

16h) conditions in Mock- and wild-type NCT (NCT-WT) stably transfected HEK293 cells. Protein load is indicated by β-tubulin analysis. B, bars represent the densitometric analysis of p53 immunoreactivity in basal conditions and are the means ± SEM of 3 independent determinations. C, p53 transcriptional activity was measured with p53 reporter gene construct (pG13-luciferase), in Mock- and NCT-WT transfectants. Bars are the means ± SE of 17 independent determinations. D, Transactivation of p53 promoter measured with the p53 promoter-luciferase construct (Pp-p53, see methods), in Mock- and wild-type NCT-WT cells. Bars are the means ± SEM of 8 independent determinations. In C and D, data have been normalized for transfection efficiencies assessed by co-transfection experiments with a β-galactosidase expression vector. E, HEK293 cells over-expressing nicastrin (NCT-WT) or not (Mock) were analysed by western blotting for phosphorylated p53 (phospho-Ser6). Actin was used as loading control. Bars represent the densitometric analysis of phospho-p53 immunoreactivity. Bars are the mean ± SE of 3 independent determinations. F, p53 or NCT were immunoprecipitated with their respective specific antibody (IP) then both p53- and NCT-like immunoreactivities were analyzed by western blots as described in the Methods. IP of p53 traps p53 (lower panel) but not NCT (upper panel). Note that IP of NCT traps NCT but also led to a very faint label at the level of p53 but that the latter was also observed after IP with preimmune serum, indicating that it was an aspecific band unrelated to p53. *p<0,01;**p<0,005; ***,p<0,0001

Fig.4: Reduction of endogenous NCT increases susceptibility to STS-induced caspase-3 activation and enhanced p53 expression and activity. A-B, Caspase-3 activity (A) and PARP immunoreactivity (B) analysed in the absence (CT) or in presence of 1μM of STS for the indicated incubation times, in $NCT^{+/+}$ and $NCT^{-/-}$ fibroblasts. Bars in A are the means ± SEM of 10 independent determinations. C,E,F, p53 immunoreactivity (C,E) and transcriptional activity (F) were analyzed as described in Methods in $NCT^{+/+}$ and $NCT^{-/-}$ fibroblasts. In E and F, bars are the means ± SEM of 7 independent determinations. D, partition of p53 expression within nuclear and cytosol compartments in $NCT^{+/+}$ or $NCT^{-/-}$ fibroblasts. G-I, SH-5Y5Y were transiently transfected with siRNA targeting NCT as described in the Methods then NCT expression (G,H) or STS-induced caspase-3 activity (I) were measured as described. In H and I, bars are the means ± SE of 4 independent determinations. *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.

Fig.5: p53 deletion abolishes NCT-induced reduction of STS-stimulated cell death. A, $p19^{arf-/-}$ and $p19^{arf-/-}$ $p53^{-/-}$ fibroblasts were treated with STS (STS, 2μM, 4h) then cell death was monitored as described in the methods. Bars correspond to the percentage of tunel-positive cells and are the means ± SEM of 17 independent fields. ***p<0,001. B-D, The indicated fibroblastic cell lines were transiently transfected with empty vector (DNA3) or nicastrin cDNA (NCT). Twenty-four hours after transfection, cells were treated with STS (2μM, 4h)

then tunel positive cells (B,C), caspase-3 activity (D) and NCT expression (C,D, upper panels) were monitored as described in the methods. Bars in C correspond to the percentage of tunel-positive cells and are the means ± SEM of 17-20 independent fields. ***p<0,001; ns, not statistically significant. Bars in D correspond to caspase-3 activity expressed as percent of that observed in $p19^{arf-/-}$ and $p19^{arf-/-}$ $p53^{-/-}$ mock-transfected cells and are the means ± SE of 7 independent experiments. **p<0.01. ns, not statistically significant.

Fig.6: NCT-associated protective phenotype is blocked by PI3K/Akt. A, MEFs fibroblasts were transfected with nicastrin using Amaxa nucleofection kit (described in methods). Over-expression of V5-tagged nicastrin was monitored by Western Blot analysis using anti-V5 antibody. Tubulin was used as loading control. B, fibroblasts were pre-treated with vehicle or LY294002 (LY, 10 μM, 30min) then incubated without or with STS (STS, 1μM, 4h). Caspase-3 activity was measured as described in the Methods. Bars are the means ± SE of 4 independent determinations. ***p<0,001; ns, non significant. C, SH-SY5Y neuroblastoma cells were transiently transfected with the indicated vector, treated with (+) or without (-) STS (1μM, 2h) then NCT, phospho-Akt and actin expressions were monitored by western blotting as described in the Methods.

Fig.7: p53 increases its own promoter transcription in fibroblasts. $p19^{arf-/-}$ and $p19^{arf-/-}$ $p53^{-/-}$ fibroblasts were transiently transfected with p53-promoter-luciferase cDNA together with either empty vector (-) or p53 (+) cDNA. p53 promoter transactivation was monitored as described in the Methods. Bars are means ± SE of 5 independent determinations. ***p<0,001.

Fig.8: The NCT-associated reduction of STS-stimulated caspase-3 activation is not affected by NCT missense and deletion mutations in HEK293 cells. A, Mock-transfected (Mock), NCT-WT, D336A/Y337A-NCT (NCT-AA), D312-340-NCT (NCT-D) cell lines were treated with 2mM of STS for16 hours then caspase-3 activity was fluorimetrically recorded as described in the Methods. Bars are the means ± SE of 10-20 independent determinations. *p<0,001 (versus Mock-transfected cells). B-D, $PS^{+/+}$ and $PS^{-/-}$ fibroblasts were transiently transfected with empty pcDNA3 vector or wild-type NCT cDNA (NCT-WT). PS1- and PS2-like immunoreactivities in $PS^{+/+}$ and $PS^{-/-}$ fibroblasts (B) and NCT expression in NCT-WT-transfected $PS^{+/+}$ and $PS^{-/-}$ (C) have been analyzed by western blot as described in Methods. Forty-eight hours after transfection, cells were treated with STS (STS, 1mM, 5h) then caspase-3 activity was fluorimetrically recorded (D). Bars in D are expressed as percent of control (STS-treated) Mock-transfected cells and represent the means ± SE of 8 independent determinations. E, HEK293 cells expressing empty pcDNA3 vector (Mock) or NCT were transiently transfected with PG13 cDNA. Twenty four hours after transfection,

cells were treated for 16 hours with DFK167 (50mM) then p53 activity was measured as described in the Methods. Bars in E are expressed as percent of control untreated Mock-transfected cells and represent the means ± SEM of 12 independent determinations. *p<0,001; **p<0,0001.

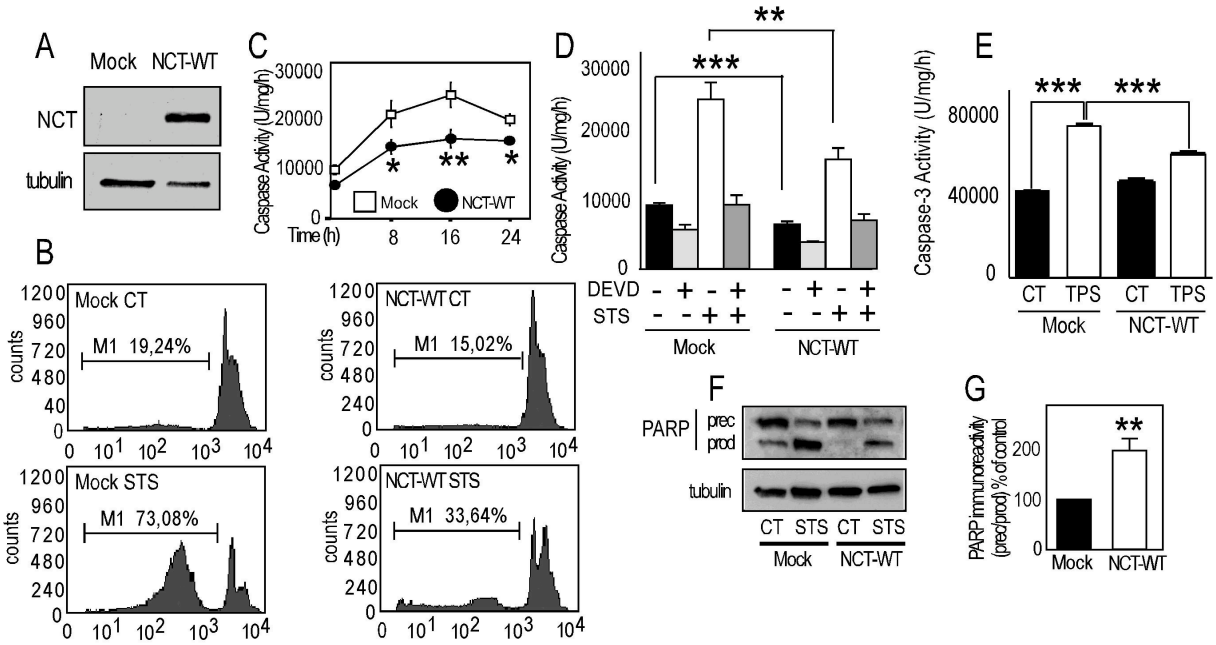
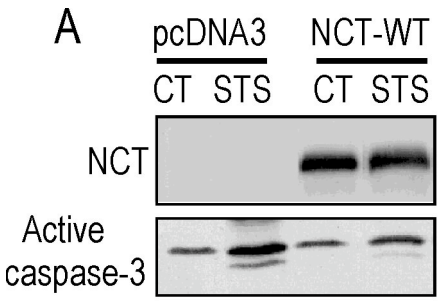
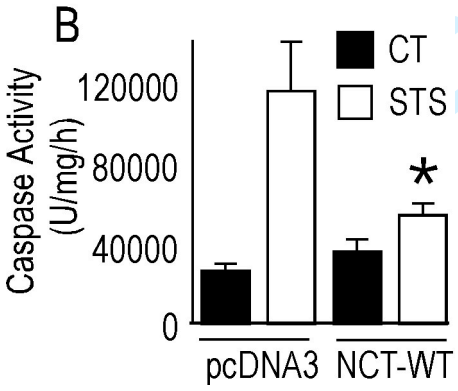


Fig.1 Pardossi-Piquard et al.





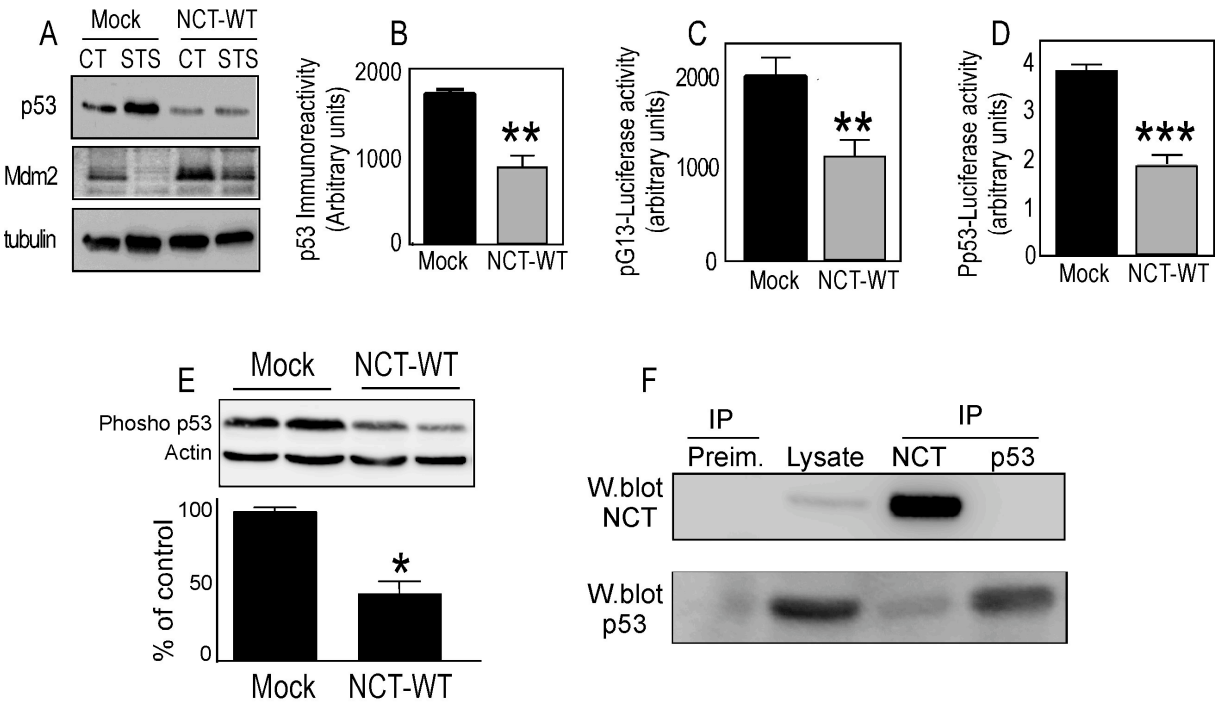


Fig.3 Pardossi-Piquard et al.

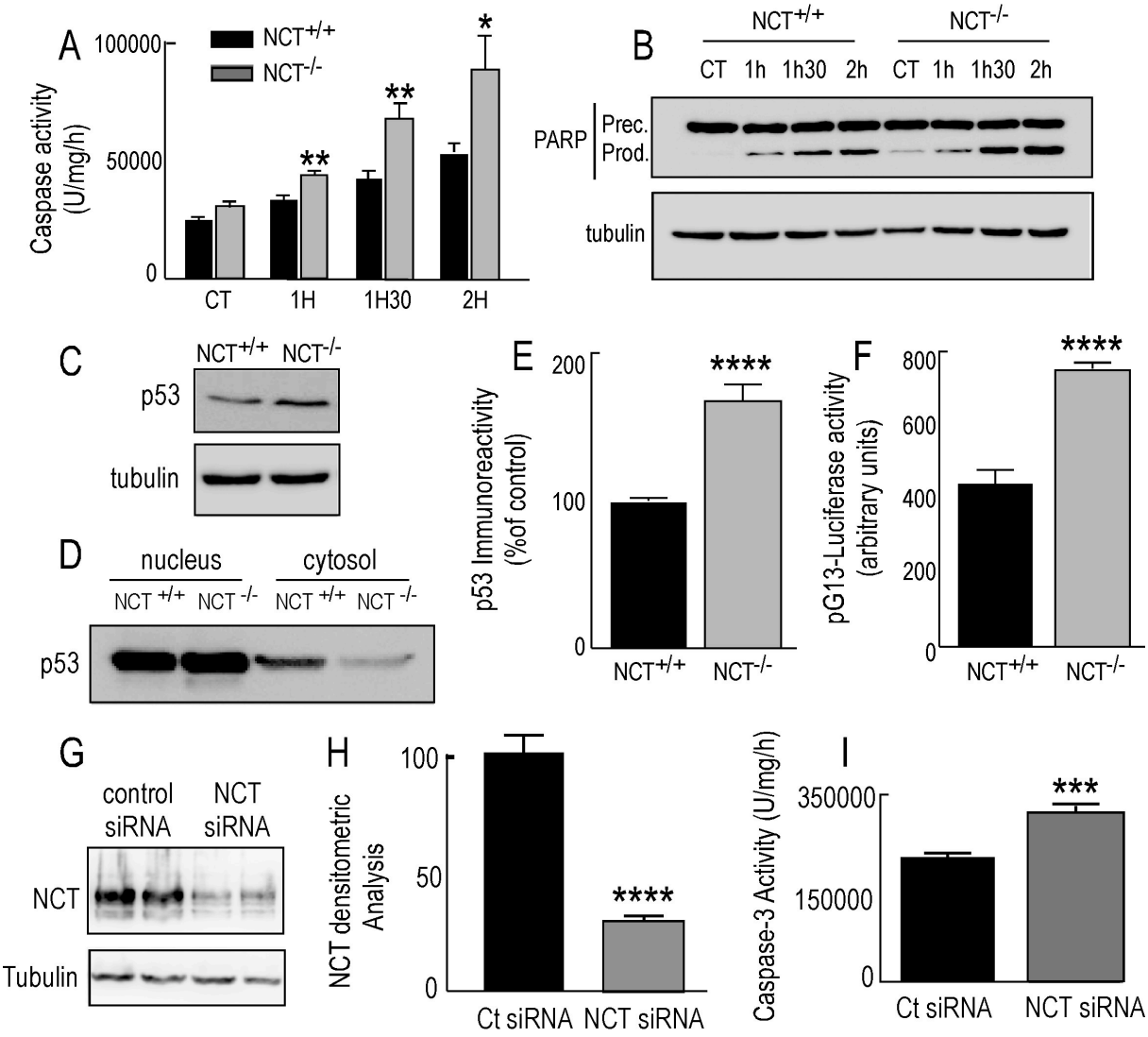


Fig.4 Pardossi-Piquard et al.

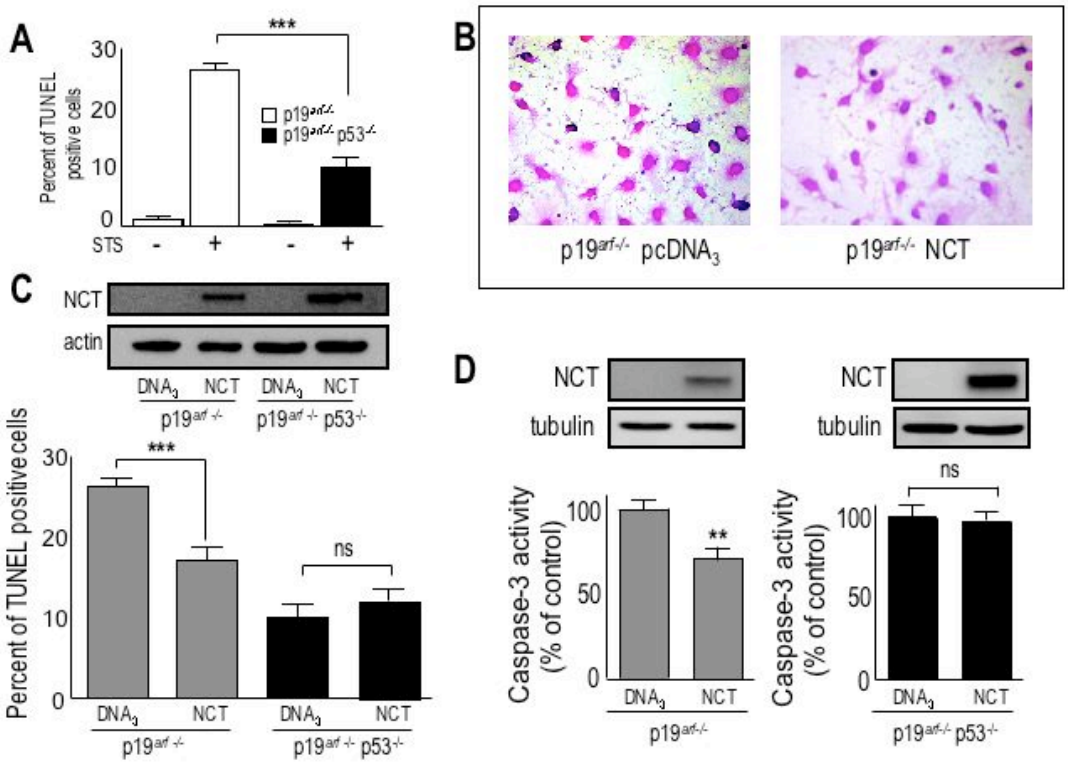


Fig. 5 Pardossi-Piquard et al.

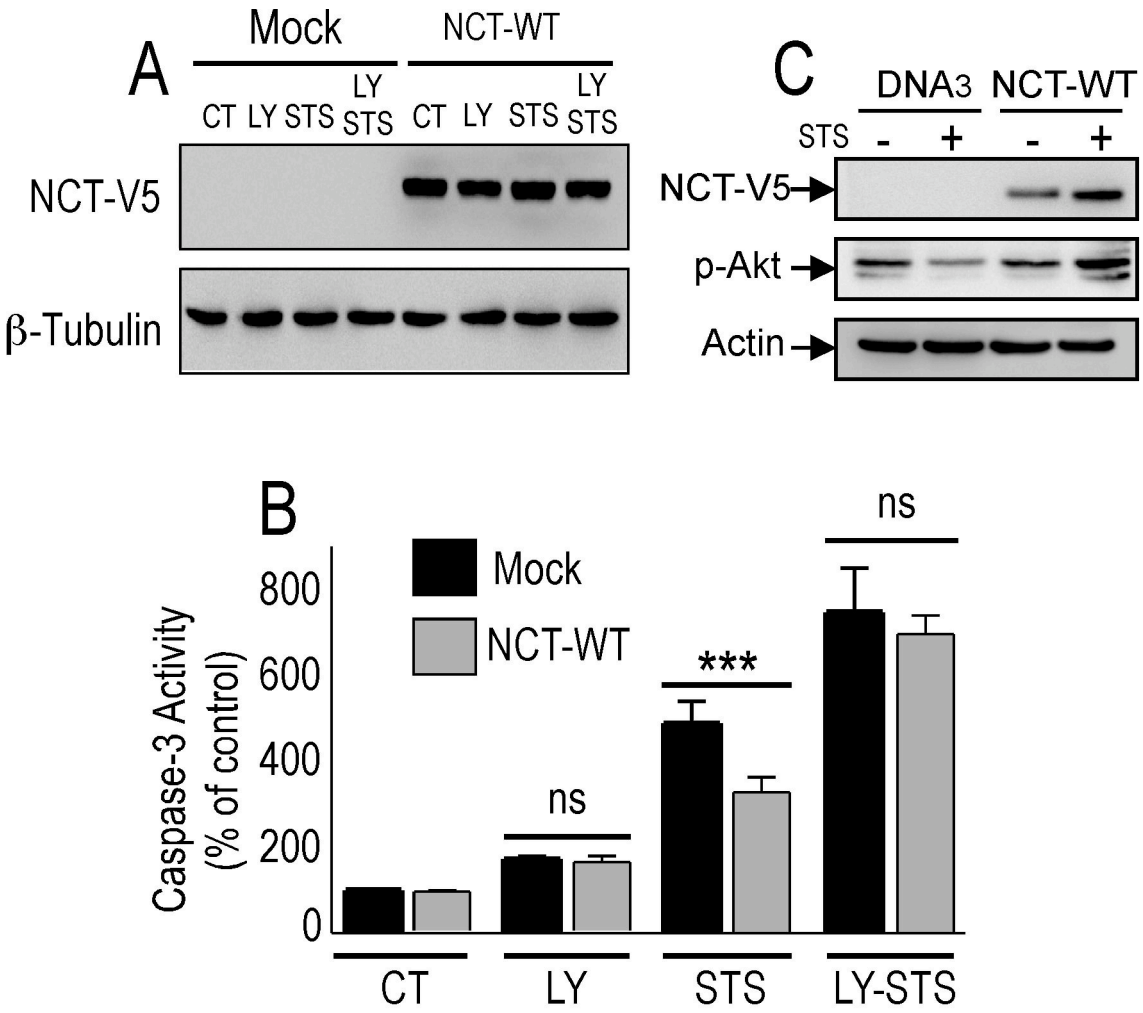


Fig.6 Pardossi-Piquard et al.

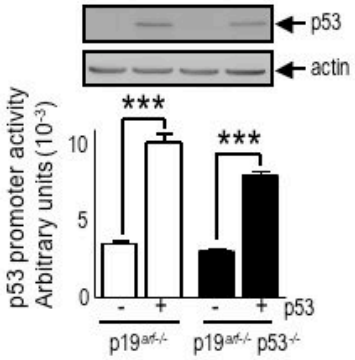


Fig.7 Pardossi-Piquard et al.

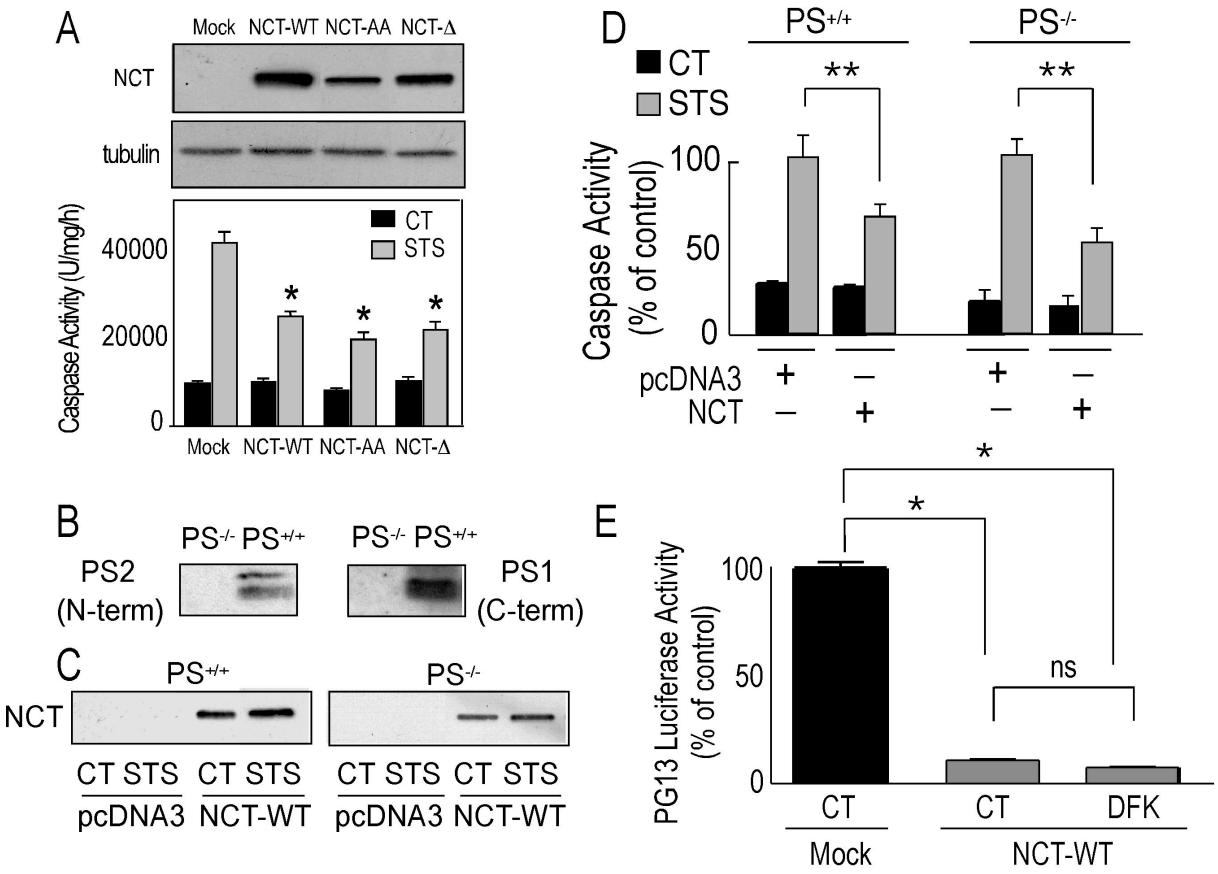


Fig.8 Pardossi-Piquard et al.

C) Discussion et Perspectives

La maladie de Parkinson tout comme la maladie d'Alzheimer est un syndrome complexe principalement de par sa multifactorialité, mais également de par l'apparition tardive des symptômes. Ce syndrome fait également partie des maladies ne touchant que l'homme compliquant ainsi le développement de modèles animaux. De plus, l'organe impliqué, le cerveau est le plus complexe et le plus difficile d'accès. Il n'existe à l'heure actuelle que des traitements palliatifs tels que la L-Dopa ou la chirurgie fonctionnelle permettant d'augmenter la durée et la qualité de vie des patients. Même si actuellement grâce aux différents modèles animaux d'énormes progrès dans la compréhension de la pathogenèse, et dans l'évolution de la maladie ont été faits il reste encore de nombreuses inconnues.

C.I) L'apoptose et la maladie de Parkinson :

Depuis plusieurs années il est admis que la neurodégénérescence observée dans la maladie de Parkinson est en partie due à l'exacerbation des processus apoptotiques. Certes la mitochondrie, la voie ubiquitine-protéasome et le stress oxydatif sont clairement identifiés comme étant impliqués dans cet emballement, cependant les signaux induisant cette série de dysfonctionnements restent encore flous. Les formes génétiques de la maladie ont permis d'identifier plusieurs protéines. Le nombre grandissant de ces protéines laisse entrevoir la multiplicité des voies impliquées dans ce syndrome, ce qui rend encore plus difficile un traitement. Cependant, ces formes ont également permis de mieux comprendre les mécanismes induisant la maladie. C'est le cas par exemple lorsque l'on s'intéresse à l'α-synucléine. En effet, il a été démontré qu'une augmentation de sa concentration que l'on peut induire par exemple en inhibant sa dégradation, entraine une abolition de sa fonciton physiologique et la mort des cellules (Alves da Costa et al., 2006).

Durant mon travail de thèse je me suis intéressée à déterminer l'influence de certaines de ces protéines sur les processus apoptotiques. De façon intéressante, tout comme l'α-synucléine, ses partenaires directes que sont la synphiline-1 et la parkine exercent des fonctions protectrices. C'est aussi le cas pour DJ-1. Ces différentes

protéines appartenant à priori à des voies différentes, DJ-1 étant plus accès sur la régulation du stress oxydatif, la parkine dans la voie ubiquitine-protéasome, protègent toutes les cellules d'un stress apoptotique. PINK1 qui elle semble liée à la mitochondrie régule également l'apoptose (Petit et al., 2005), c'est aussi le cas de la dardarine (LRRK2) qui elle est une kinase (Smith et al., 2005b). Cette capacité à réguler l'apoptose est abrogée pour toutes ces protéines par les mutations pathogènes liées à la maladie. Donc en plus de leur fonction propre par exemple d'ubiquitine ligase ou de facteur de transcription pour la parkine, ces protéines exercent un rôle dans les mécanismes apoptotiques. Cependant, il reste à déterminer pour certaines les cascades spécifiques dans lesquelles elles interviennent mais également les liens entre ces voies.

C.II) L'activité régulatrice des caspases effectrices :

Nos différentes études, en particulier les travaux portant sur les fonctions de la synphiline-1 et de DJ-1, ont mis en évidence une régulation de ces deux protéines par les caspases effectrices. En effet, la caspase-3 pour la synphiline-1 et la caspase-6 pour DJ-1 clivent ces protéines suite à un stress afin de produire des fragments C-terminaux capables de réguler leurs activations. Nous avons donc mis en évidence l'importance des caspases dans la régulation des mécanismes liés au développement de la maladie et, en particulier la présence de rétrocontrôles négatifs ayant pour but de protéger les cellules des effets des caspases effectrices en diminuant leurs activités. Ces deux protéines ne sont pas les seules à être clivée par les caspases, en effet, la parkine peut également subir un clivage par les caspases-1 et -8. Cependant, ce clivage contrairement à ceux sur la synphiline-1 et sur DJ-1 n'induit pas de phénotype protecteur mais inversement conduit à l'inactivation de la parkine par perte de son activité ubiquitine ligase et à la mort cellulaire (Kahns et al., 2003; Kahns et al., 2002). De façon intéressante une autre protéine la huntingtine impliquée dans la maladie de Huntington une autre maladie neurodégénérative, subit également un clivage par la caspase-6, conduisant à une translocation de la huntingtine dans le

noyau. Contrairement au clivage de DJ-1, protecteur, celui de la huntingtine induit les dysfonctions et la dégénérescence neuronales (Graham et al., 2006; Sawa et al., 2005; Warby et al., 2008).

Depuis quelques années plusieurs mutations ont été découvertes sur le gène de DJ-1, en particulier la mutation D149A (Takahashi-Niki et al., 2004). Nous avons déterminé que cette mutation se situe sur le site de clivage de la caspase-6, inhibant ainsi ce clivage et donc la fonction protectrice du fragment. Nous avons démontré que DJ-1 protège les cellules de la mort cellulaire en séquestrant p53 et, en activant la voie Akt. Le clivage de la huntingtine par la caspase-6 conduit à sa translocation dans le noyau, il serait intéressant d'envisager que le clivage de DJ-1 produisant un fragment C-terminal conduit également à la translocation de celui-ci dans le noyau, essentiel à la fonction de DJ-1. On pourrait alors envisager que ce fragment issu de DJ-1 soit un facteur de transcription.

C.III) p53 à la croisée des chemins :

Autre fait intéressant, malgré la multiplicité des protéines et des voies impliquées dans la pathogenèse de la maladie de Parkinson, une protéine semble être un point de convergence pour plusieurs de ces routes. En effet, le facteur de transcription p53 apparait de façon récurrente dans les voies régulées par les protéines impliquées dans les formes familiales de la maladie. Ce facteur de transcription a également été mis en exergue dans de nombreuses études mettant à profit les différents modèles utilisant les toxines (Duan et al., 2002; Eberhardt and Schulz, 2003). Nous avons démontré que les fonctions protectrices de la synphiline-1, de DJ-1 et de la parkine passent par la modulation de la voie dépendante de p53. Le Dr Alves da Costa a démontré qu'il en est de même pour l'α-synucléine (Alves da Costa). Cependant, suivant la protéine impliquée, la modulation de cette voie à lieu à différents niveaux. DJ-1 est capable de séquestrer p53 dans le cytoplasme empêchant ainsi l'auto transactivation de son promoteur, et/ou d'activer la voie Akt favorisant

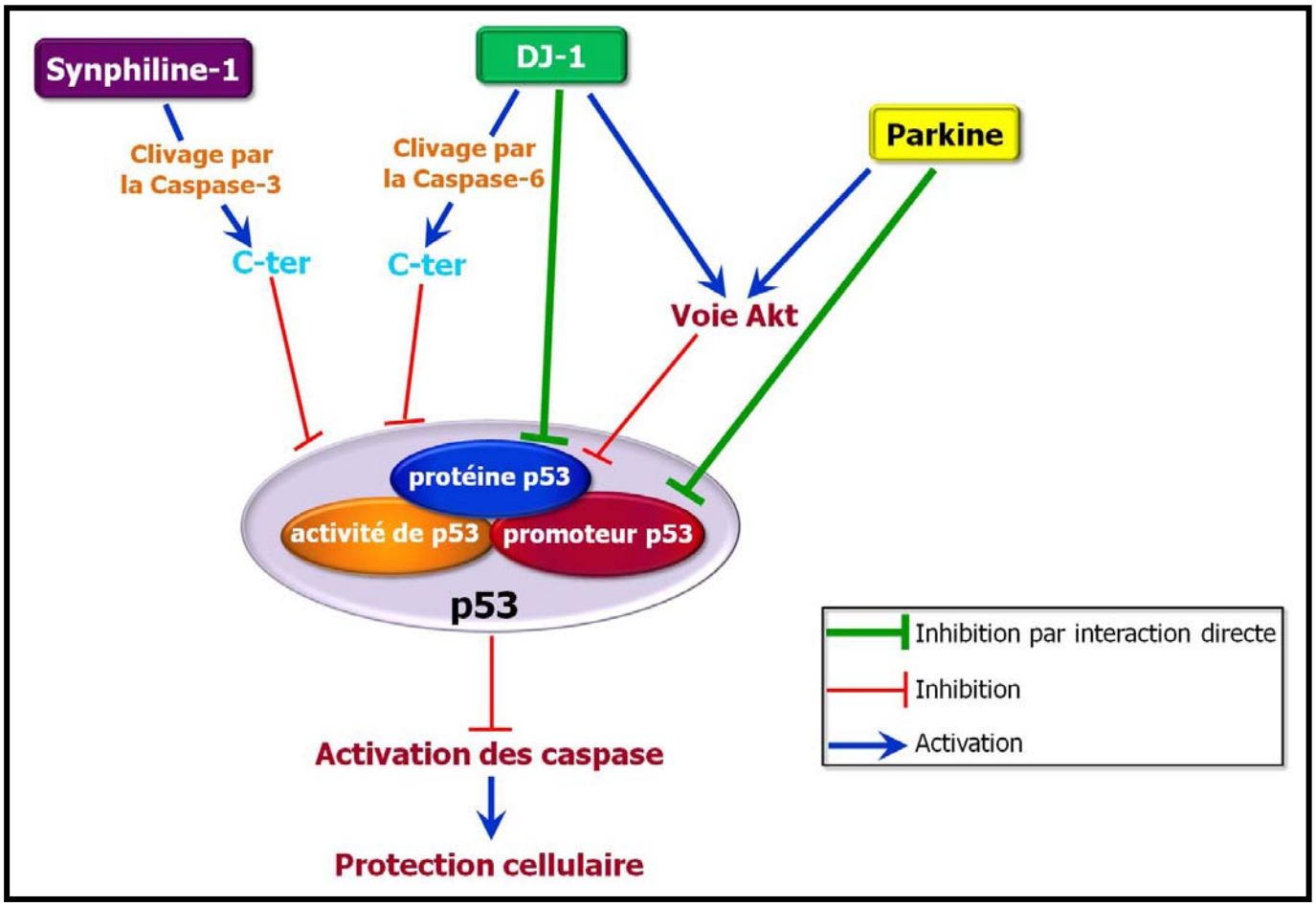


Figure 42 : Schéma récapitulant les mécanismes et intermédiaires liés à la fonction protectrice de la synphiline-1, de DJ-1 et de la parkine

Cette figure illustre et regroupe les mécanismes et les intermédiaires intervenant dans la fonction protectrice de la synphiline-1, de DJ-1 et de la parkine, que j'ai identifiés durant ces trois dernières années. La fonction protectrice de la synphiline-1 passant par l'inhibition de la voie p53 est protée par son fragment C-terminal issu de son clivage par la caspase-3. Celle de DJ-1 passe aussi par l'inhibition de p53 est portée par son fragment C-terminal issu de son clivage par la caspase-6, mais DJ-1 est aussi capable de réguler p53 de façon directe en le séquestrant dans le cytoplasme et en activant la voie Akt. Quand à la parkine elle active également la voie Akt, mais régule aussi p53 en se fixant directement sur son promoteur et en inhibant sa transactivation, grâce à sa fonction de facteur de transcription.

ainsi la dégradation de p53 par Mdm2. De plus, nous avons identifié une nouvelle fonction de la parkine. En effet, celle-ci est capable de réguler négativement l'activité du promoteur de p53 en se fixant directement dessus, la parkine est donc un nouveau facteur de transcription. Dans cette étude, le gel shift nous montre que la parkine se fixe bien sur le promoteur de p53, à l'aide des différentes constructions nous avons limité la taille du fragment sur lequel la parkine se fixe (Figure 42). Cependant, il serait intéressant de déterminer la séquence cible de la parkine. On sait que la parkine régule de nombreux gènes par sont activité ubiquitine ligase, nous avons déterminé que la parkine possède également une fonction de facteur de transcription, la parkine ne pourrait-elle pas réguler ces mêmes gènes à un niveau transcriptionnel en réprimant ou en activant leur promoteur (Ko et al., 2006; Um et al., 2006; Zhang et al., 2000). L'alignement des séquences des différentes cibles de la parkine pourrait nous permettre de déterminer la séquence de ce site.

Au laboratoire nous avons pu observer que p53 est également une protéine centrale dans la pathogenèse de la maladie d'Alzheimer. En effet nous avons montré qu'outre son rôle dans l'induction de l'apoptose, p53 est capable de réguler l'expression de membres du complexe γ-secrétase comme Pen-2. Ce facteur de transcription est aussi impliqué dans la maladie de Huntington où il est capable de réguler positivement la huntingtine (Feng et al., 2006). L'oncogène p53 est donc une protéine importante dans de nombreuses maladies neurodégénératives.

C.IV) Plusieurs voies mais aussi des ponts :

De nombreuses protéines sont impliquées dans la pathogénèse de la maladie de Parkinson, elles sont de plus impliquées dans des voies différentes liées à la mitochondrie, au protéasome ou encore au stress oxydatif, elles possèdent à priori des fonctions différentes, cependant il existe des ponts reliant ces voies. Nous avons pu établir que les observations faites par l'équipe du Dr Moore sur la modulation de la stabilité de DJ-1 par la parkine sont un de ces ponts. En effet, nous avons montré

que par l'intermédiaire de cette protéine centrale qu'est p53, la parkine a la capacité de faire varier l'expression de DJ-1. De façon intéressante, plusieurs études ont également montré un lien entre PINK1 et la parkine, ces deux protéines font parti de la même voie de signalisation. Dans cette voie PINK1 agit en aval de la parkine. En effet, il a été montré que la déplétion de PINK1 peut être compensée par la transfection de la parkine. Une étude a démontré que la parkine est également capable d'interagir avec LRRK2. De plus, la surexpression de la parkine induit une augmentation du nombre d'agrégats dans les cellules exprimant la dardarine, ces inclusions pouvant contenir la dardarine et la parkine, ainsi qu'une augmentation de l'ubiquitinylation des protéines agrégées (Smith et al., 2005b). Malgré la multiplicité des voies qui sont impliquées compliquant énormément les traitements, comme on la vu il existe des ponts entres ces voies mais également un point commun p53 cette protéine pourrait être une cible thérapeutique à envisager. Des études ont d'ailleurs déjà été menées et se poursuivent dans ce sens à l'aide d'inhibiteurs de p53 comme la pifithrine injecté dans le cerveau de souris modèles (Duan et al., 2002; Rio and Velez-Pardo, 2008).

En plus de la voie p53, la voie PI3kinase/Akt semble également un point commun à la fonction protectrice de plusieurs des protéines impliquées dans les formes familiales de la maladie : DJ-1, parkine (Yang et al., 2005). En outre, la voie Akt se trouve en amont de p53, agir au niveau de cette voie pourrait permettre de mieux moduler p53 pour intervenir uniquement sur sa fonction dans la survie cellulaire et ne pas interférer dans les autres. La modulation d'une voie importante comme l'est la voie Akt et plus précisément une activation de la voie Akt pourrait permettre de protéger les neurones dopaminergiques. Cette voie est en effet essentielle dans la neuroprotection induite par exemple par les œstrogènes ou la β-synucléine (Hashimoto et al., 2004; Quesada et al., 2008). Une étude récente a étudié la variabilité du gène *AKT1* comme facteur de risque pour la maladie de Parkinson. Ils ont montré que certains haplotypes pourraient réduire les risques de développer la maladie, confirmant ainsi le potentiel de cette cible en thérapie (Xiromerisiou et al., 2008).

D) Annexes

Liste des publications

Giaime E., Sunyach C., Herrant M., Grosso S., Auberger P., McLean P., Checler F., and Alves da Costa C. Caspase-3-derived C-terminal product of synphilin-1 displays antiapoptotic function via modulation of p53-dependent cell death pathway. *J. Biol. Chem* 2006 Apr 28; 281(17):11515-11522.

Dunys J., Kawarai T., **Giaime E.**, Wilk S., Herrant M., Auberger P., St George-Hyslop P., Alves da Costa C., Checler F. Study on the putative contribution of caspases and the proteasome to the degradation of Aph-1 and Pen-2. *Neurodegener Dis.* 2007; 4(2-3):156-63.

Alves da Costa C, **Giaime E.**, West A., Corti O, Brice A, Abou-Sleiman P.M., Wood N.W., Takahashi H, Goldberg M.S, Shen J, and Checler F. Parkin-induced transcriptional repression of p53 is impaired by Parkinson's disease-associated mutations. En révision.

Giaime E., Sunyach C., Druon C., Robert G., Grosso S, Auberger P., Goldberg M.S., Shen J., Heutink P., Pouysségur J., Pagès G., Checler F., and Alves da Costa C. Loss of function of DJ-1 triggered by Parkinson's diseaseassociated mutation is due to proteolytic resistance to caspase-6. En révision.

Giaime E., Sunyach C., Druon C., Corti O., Brice A., Heutink P., Dawson T., Ariga H., Checler F., and Alves da Costa C. Ubiquitin ligase independent and p53-mediated modulation of DJ-1 by parkin implication in Parkinson's disease. En préparation.

Pardossi-Piquard R., Dunys J., **Giaime E**., Guillot-Sestier M-V, St. George-Hyslop P., Alves da Costa C., and Checler F. p53-dependent control of cell death by nicastrin: lack of requirement for presenilin-dependent γ-secretase complex. Soumis pour publication.

Dunys J., Sevalle J., **Giaime E**., Vitek M., Renbaum P., Levy-Lahad E., Zhang Y-W., Xu H., Alves da Costa C., Checler F. p53-dependent control of pen-2 promoter transcription by presenilins and evidence of a feed-back control of presenilin 1 and 2 transactivation by pen-2. En révision.

Soehn A.S., Franck T., Biskup S., Floss T., Trang P., Vogt Weisenhorm D.M., **Giaime E.**, Cebo D., Berg D., Melle C., Strauss K.M., Rott R., Engelender S., Kalbacher H., Ott E., Tomiuk J., Von Eggeling F., Pahnke J., Meitinger T., Aho S., Krüger R., Alves da Costa C., Wurst W., Gasser T., Riess O. "Periphilin is a novel interactor of synphilin-1, a protein implicated in Parkinson's disease". En révision.

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Abréviations

6-OHDA	6-hydroxydopamine
Aβ	Amyloid-β-peptide
AICD	APP Intracellular Domain
APP	Amyloid Precursor Protein
Aph-1	Anterior Pharynx defective-1
Bcl-2	B-cell lymphoma 2
DAT	Dopaminergic Transporter
ERK	Extracellular signal-Related Kinase
GAPDH	Glycéraldéhyde-3-phospate déshydrogénase
HEK293	Human Embryonic Kidney 293
LRRK2	Leucine Rich Repeat Kinase 2
Mdm2	Mouse Double Minute 2
MPP	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MEF	Mouse Embryonic Fibroblasts
NCT	Nicastrine
PARP	Poly-ADP-Ribose-Polymerase
Pen-2	Presenilin enhancer-2
PI	Point isoelectrique
PI3K	PhosphoInositol-3 Kinase
PINK1	PTEN-Induced Kinase1
PTEN	Phosphatase and TENsin homolog
ROS	Reactive Oxygen Species
SNpc	Substance Noire pars compacta
STS	Staurosporine
TSM1	Telencephalon Specifc Murine 1
TUNEL	Terminal dUTP Nick End Labeling
UCHL1	Ubiquitin Carboxy Hydroxylase 1

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Résumé

La maladie de Parkinson est un syndrome neurodégénératif, caractérisé d'un point de vue histopathologique par une dégénérescence spécifique des neurones dopaminergiques de la substance noire. Elle peut être d'origine sporadique ou génétique. Les formes familiales résultent de mutations portées par différentes protéines : la parkine, DJ-1, PINK1, l'α-synucléïne, UCHL1, et LRRK2. Ces mutations s'accompagnent d'un dysfonctionnement du système ubiquitine-protéasome, d'un défaut mitochondrial ainsi que d'une augmentation du stress oxydatif conduisant à la mort neuronale par apoptose. La dégénérescence des neurones dopaminergiques est associée à l'apparition d'agrégats fibrillaires nommés corps de Lewy. Ces inclusions cytoplasmiques sont principalement composées d'α-synucléïne. Au cours de mon travail de thèse, je me suis intéressée à la synphiline-1, un partenaire de l'α-synucléïne, et à deux protéines majeures impliquées dans les formes récessives de la maladie de Parkinson, DJ-1 et la parkine.

Je me suis consacrée à l'étude de leurs fonctions physiologiques, et plus particulièrement à leurs implications dans les processus de mort cellulaire par apoptose. Ainsi, j'ai déterminé que ces protéines réduisent l'activité de la caspase-3 induite par différents stimuli. Cette fonction protectrice passe par la régulation de la voie dépendante de l'oncogène p53. De plus, j'ai identifié DJ-1 et la synphiline-1 comme étant substrats des caspases, et également que les fragments C-terminaux issus de ce clivage portent leurs activités biologiques. De façon intéressante, différentes mutations pathogènes portées par DJ-1 et la parkine conduisent à une perte de fonction.

Parallèlement, j'ai étudié des aspects de la régulation transcriptionnelle de DJ-1 et de la parkine par le facteur de transcription p53. J'ai mis en évidence une boucle de régulation entre p53, DJ-1 et la parkine. En effet, j'ai montré que ces deux protéines sont capables de réguler l'expression de p53. De plus, j'ai déterminé que la parkine régule positivement DJ-1, et que ce contrôle s'effectue via la régulation transcriptionnelle de DJ-1 par p53.